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Award Number: DAMD17-99-1-9479

TITLE: The Nigrostriatal Dopamine System and Methamphetamine: Roles for Excitotoxicity and Environmental, Metabolic and Oxidative Stress

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REPORT DATE: July 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-07-2005	Final	1 Jul 1999 - 14 Jun 2005

4. TITLE AND SUBTITLE
The Nigrostriatal Dopamine System and Methamphetamine: Roles for Excitotoxicity and Environmental, Metabolic and Oxidative Stress

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-99-1-9479

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Bryan Yamamoto, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Boston University
Boston, MA 02118

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT – **SEE ATTACHED PAGE**

15. SUBJECT TERMS
Neurotoxin

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	176	

Abstract

Degeneration of the nigrostriatal dopamine system is linked to the pathophysiology of Parkinson's disease. Similarly, the psychostimulant drug, methamphetamine also produces relatively selective damage to nigrostriatal dopamine neurons and is a widespread problem and drug of abuse throughout the U.S. However, the neurochemical underpinnings that mediate methamphetamine toxicity and Parkinson's disease are unknown.

Several variables common to methamphetamine toxicity and Parkinson's disease, each of which may be important but alone are insufficient, may account for the neurodegeneration of the nigrostriatal dopamine path. It is hypothesized that the convergence of excitotoxicity, free radicals and a depleted bioenergetic state produces damage to dopamine neurons. Moreover, environmental stressors, which also increase free radicals and excitatory amino acids predispose dopamine neurons to damage. Consequently, environmental stress may be synergistic with oxidative and metabolic insults as well as glutamate to culminate in dopamine cell death. ***The major objective is to examine the interaction between environmental stress and methamphetamine and the convergent action of excitotoxicity and bioenergetic and oxidative stress to produce damage to nigrostriatal dopamine neurons.*** A multidisciplinary approach will be used as well as pharmacological strategies that we posit to be neuroprotective against methamphetamine, excitotoxicity, and bioenergetic and oxidative stress will be examined.

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INTRODUCTION

Degeneration of the nigrostriatal dopamine system is linked to the pathophysiology of Parkinson's disease. Similarly, the psychostimulant drug, methamphetamine, also produces relatively selective damage to nigrostriatal dopamine neurons and is rapidly becoming a widespread problem and drug of abuse throughout the U.S. However, the neurochemical underpinnings that mediate methamphetamine toxicity and Parkinson's disease have escaped definition.

We propose that several variables common to methamphetamine toxicity and Parkinson's disease, each of which may be important but alone are insufficient, account for the neurodegeneration of the nigrostriatal dopamine path. It is hypothesized that the convergence of excitotoxicity, free radicals and a depleted bioenergetic state produces damage to dopamine neurons. Moreover, environmental stressors, which also increase free radicals, excitatory amino acids, and alter energy metabolism, predispose dopamine neurons to damage. Consequently, environmental stress may be synergistic with oxidative and metabolic insults as well as glutamate to culminate in dopamine cell death. *The major objective is to examine in rats the interaction between environmental stress and methamphetamine and the convergent action of excitotoxicity, bioenergetic stress, and oxidative stress to produce damage to nigrostriatal dopamine neurons.* A multidisciplinary approach of *in vivo* and *in vitro* biochemical and histochemical methods will be used. In addition, pharmacological strategies that we posit to be neuroprotective against methamphetamine, excitotoxicity, and bioenergetic and oxidative stress will be examined.

FINAL PROGRESS REPORT

Per the instructions for "Reporting Requirements", appended publications are substituted for detailed descriptions related to the rationale, methodology, illustrations of the results and statistical analyses. References to these publications as Appendices are in the body of the report. Literature citations in the Body are referenced in the respective Appendix.

Body:

OBJECTIVE 1:

To examine the interactions between methamphetamine, environmental stress and excitotoxicity

Introduction:

There is increasing evidence that repeated exposure to environmental stressors can alter behavioral (self-administration, locomotor activity) and neurochemical (dopamine release) responses to drugs of abuse. However, it is unknown if exposure to stress enhances the vulnerability of the dopamine system to the neurotoxic effects of drugs of abuse such as methamphetamine. The long-term neurotoxic effects of METH are evidenced by long-term decreases in markers of

dopamine transmission such as a depletion of dopamine content. In addition, hyperthermia is known to mediate in part, the neurotoxic effects of METH.

The hypothesis of this series of studies was that prior exposure to chronic unpredictable stress will enhance the acute effects of METH as evidenced by the release of dopamine and glutamate as well as the hyperthermic responses to METH. Furthermore, it was hypothesized that exposure to unpredictable stress will enhance the long-term depletions of dopamine.

Results:
The following results have been published (*Neuroscience* 124: 637-46, 2004; *Psychopharmacology* 169: 169-175, 2003) and noted in **Appendix 1 and Appendix 2, respectively. The figure numbers are related to the illustration numbers in the appendix.**
Appendix 1: Ten days of unpredictable stress 1) augmented the acute increase in extracellular striatal dopamine concentrations in response to injections of 7.5 (Appendix 1, Fig. 2A; $p<0.05$ $F(9,126)=49.9$, ANOVA) or 10 mg/kg METH (Appendix 1, Fig. 2B; $p<0.05$ $F(9,90)=35.02$, ANOVA and 2) produced greater depletions of striatal dopamine 7 days following the injection regimen (Figs. 3A and B; $p<0.05$ ANOVA) of either 7.5 or 10 mg/kg METH, respectively compared to non-stressed rats administered METH.

Appendix 2: Chronic unpredictable stress also augmented the acute hyperthermic response to METH. These results have been published (Psychopharmacology 169: 169-175, 2003) and noted in **Appendix 2**.
Stressed and non-stressed control rats did not differ in body weight at the start of the stress protocol [stressed: 213.1 ± 2.6 g; controls: 215.2 ± 2.7 g; $t(94)=0.24$]. However, by the test day (day 11), control rats weighed more than chronically stressed rats [stressed: 274.3 ± 2.7 g; controls: 301.1 ± 2.9 g; $t(94)=6.7$, $P<0.01$].

Systemic injection of 1.5 mg/kg DOI significantly increased rectal temperature in both stressed and control rats over time [2 days of stress: $F(4,40)=33.5$, $P<0.01$ (ANOVA); 10 days of stress: $F(6,150)=69.93$, $P<0.01$, ANOVA]. This effect of DOI compared to saline injected rats was confirmed by the AUC data for rats exposed to 10 days of chronic stress [Fig. 1, $F(1,18)=25.5$, $P<0.01$] and non-stressed control rats [$F(1,19)=28.6$, $P<0.01$, ANOVA).

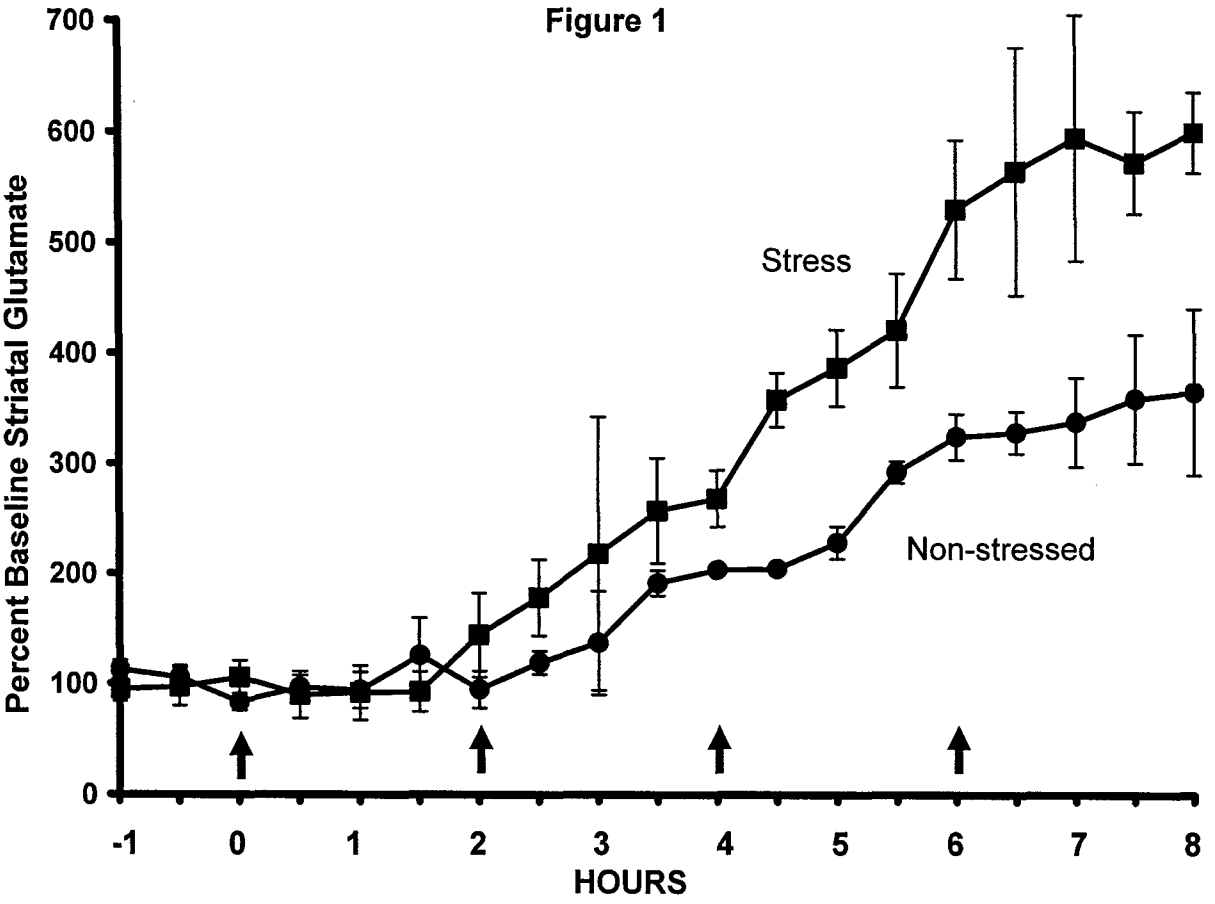
The 5-HT2 receptor antagonist LY-53,587 attenuated the DOI-stimulated hyperthermic response of stressed [Fig. 2, $F(18,228)=7.1$, $P<0.01$) and control rats [Fig. 3, $F(18,234)=7.7$, $P<0.01$]. Both stressed and control rats injected with vehicle and DOI had significantly higher rectal temperatures than rats injected with LY-53,587 and saline, or LY-53,857 and DOI [AUC stressed: $F(3,38)=14.6$; AUC control: $F(3,39)=13.7$].

Moreover, the enhanced hyperthermic response to DOI in chronically stressed rats persisted for 8, 30 and 60 days after the exposure to chronic stress. That is, DOI significantly increased body temperature compared to pre-injection rectal temperatures on all test days [$F(5,390)=276.15$, $P<0.01$]. A similar effect was

observed when each test day was analyzed separately [day 8: $F(5,125)=209.8$, $P<0.01$; day 30: $F(5,75)=96.7$, $P<0.01$; day 60: $F(5,100)=64.9$, $P<0.01$]. Stressed rats had significantly higher rectal temperatures compared to non-stressed controls on all test days [Fig. 4, AUC $F(1,60)=11.1$, $P<0.01$]. These differences between stressed and control rats were also evident when each day was analyzed separately [Table 2, day 8: $F(5,125)=3.1$, $P<0.05$; day 30: $F(5,75)=3.1$, $P=0.05$; day 60: $F(5,100)=3.6$, $P<0.05$].

Figure 1 below (*new data since submission of the Final Report*) shows that prior exposure to chronic unpredictable stress enhanced the METH-induced increases in extracellular glutamate within the striatum ($p<0.05$; Overall time X stress group interaction effect, n=11/group) (Arrows indicate time of injection of METH (7.5 mg/kg ip).

Figure 1



Discussion (Citations are referenced in the bibliography of the Appendix):

Several factors proposed to mediate dopamine depletions following high doses of METH may be responsible for the observed potentiated depletions of dopamine in chronically stressed rats. A long-term depletion of dopamine content in the striatum after METH is correlated with elevated body temperatures during METH administration (Itoh et al., 1986; Bowyer et al., 1994). Pharmacological agents

that lower body temperature attenuate METH-induced dopamine depletions in the striatum, as do lower ambient temperatures (Sonsalla et al., 1991; Bowyer et al., 1992, 1994). In the present study, stressed rats showed greater hyperthermia during METH administration (Fig. 1) and this increase in rectal temperature may contribute to the enhanced dopamine damage observed 1 week after METH injections (Fig. 3). Alternatively, chronic stress may enhance METH-induced dopamine depletions in the striatum by increasing the acute release of dopamine. Extracellular dopamine concentrations were greater following METH injections in rats exposed to unpredictable stress, than in non-stressed controls (Fig. 2). Blocking dopamine transmission through inhibition of synthesis, blockade of transporter-mediated uptake or co-administration of dopamine antagonists attenuates METH-induced dopamine depletions (Buening and Gibb, 1974; Schmidt et al., 1985; Sonsalla et al., 1986; Marek et al., 1990; Pu et al., 1994).

The acute increase in extracellular dopamine and/or glutamate may contribute to longer-term dopamine depletions through the generation of free radical. The potentiation of METH-induced extracellular dopamine levels in rats exposed to stress parallels other findings following a challenge injection of amphetamine or cocaine (for review see Kalivas and Stewart, 1991). Repeated exposure to stress may contribute to the enhanced dopamine release by increasing tyrosine hydroxylase and/or the releasable stores of dopamine, inhibiting dopamine catabolism, decreasing dopamine uptake, or increasing impulse generation in dopaminergic neurons. Ortiz and colleagues (1996) reported an increase of tyrosine hydroxylase in the ventral tegmental area, but not the substantia nigra, following the same 10-day stress procedure as used for the current study. Although increases in dopamine synthesis may explain elevated extracellular dopamine concentrations in the mesolimbic terminal regions, such as the nucleus accumbens, other mechanisms may be operative in the nigrostriatal system (Beitner-Johnson et al., 1991, 1992; Beitner-Johnson and Nestler, 1991; Sorg and Kalivas, 1991).

Stress is known to increase the release of glutamate and we have shown previously that METH will also increase glutamate release. Therefore, the combination of stress and METH may be additive on the stimulation of glutamate to eventually produced excittoxicity. However, it is not known if METH produces evidence of excitotoxicity (but see Objective 7).

Due to the ability of METH to release dopamine through reverse transport (Fischer and Cho, 1979), stress-induced alterations in the DAT may account for the augmented release of dopamine during METH injections. However, acute social stress in mice housed in isolation reduced DAT binding in the striatum (Isovich et al., 2001), as did exposure of male tree shrews to chronic subordinate stress (Isovich et al., 2001). These studies suggest that the observed increase in striatal extracellular dopamine in chronically stressed rats is not due to increases in DAT. Interestingly, elevated body temperature also can influence the function of DAT by increasing the intracellular accumulation of METH (Metzger et al., 2000; Xie et al., 2000). Therefore, the augmented hyperthermic responses

in the stressed rats (Fig. 1, Appendix 2) may enhance the function of DAT and subsequently contribute to observed increases in extracellular dopamine during METH treatment, irrespective of the number of transporters. The acute increases or delayed depletions of dopamine observed in the rats exposed to chronic stress do not appear to be due to increased bioavailability of METH in the striatum. METH concentrations in the striatum of stressed and non-stressed rats were similar throughout the METH injection regimen (Fig. 4). While METH concentrations measured with *in vivo* microdialysis suggest that the extracellular concentrations are similar between stressed and non-stressed rats, this technique does not assess the concentrations of METH in dopamine terminals. The concentration of METH in the terminals and subsequent alterations of vesicular pH gradients may be more critical to the longer-term dopamine depletions than extracellular METH concentrations (Sulzer and Rayport, 1990).

Overall, several mechanisms may contribute to the acute increases in hyperthermia or extracellular dopamine in the striatum and the potentiated decreases in dopamine tissue content. The precise effects of repeated, unpredictable stress on the brain are unknown but alterations in 5-HT receptors (Ossowska et al., 2001) or the dopaminergic system (Ortiz et al., 1996; Ossowska et al., 2001), may account for the enhanced hyperthermia, mortality, extracellular dopamine concentrations, or depletions of dopamine content in the striatum. Due to the broad overlap of stress and drug use (Piazza and LeMoal, 1998; Yui et al., 1999, 2001; Koob and LeMoal, 2001), the increased vulnerability of the brain by exposure to unpredictable stressors may be important for understanding the potential detrimental effects of drugs of abuse.

Objective 2:
To further examine the effects of chronic stress on basal concentrations of glutamate by measuring extracellular glutamate in the hippocampus.

Introduction
Evidence is accumulating that stress is associated with the onset of depression, a dysregulation of the hypothalamic-pituitary-adrenal axis, and possible neurodegeneration. With regard to the latter, McEwen and colleagues have described a model of stress-induced morphological reorganization in the hippocampus that appears to be mediated by excitatory amino acids and adrenal steroids (McEwen, 1997). Unpublished observations indicate that chronic stress increases mRNA expression of the glial glutamate transporter (GLT-1) in the CA3 region of the hippocampus (Reagan and McEwen) and further support the role of excitatory amino acids in mediating the neurodegeneration observed in this area following chronic stress. Consistent with these findings of stress-induced increases in excitatory amino acid transmission in the hippocampus, we have shown that acute restraint stress increases hippocampal glutamate release measured in vivo; an effect that is reversed by adrenalectomy (Lowy et al., 1993). Therefore, we hypothesized the chronic unpredictable stress will

increase the extracellular concentrations of glutamate in the hippocampus as measured by *in vivo* microdialysis.

Results:

Figure 2

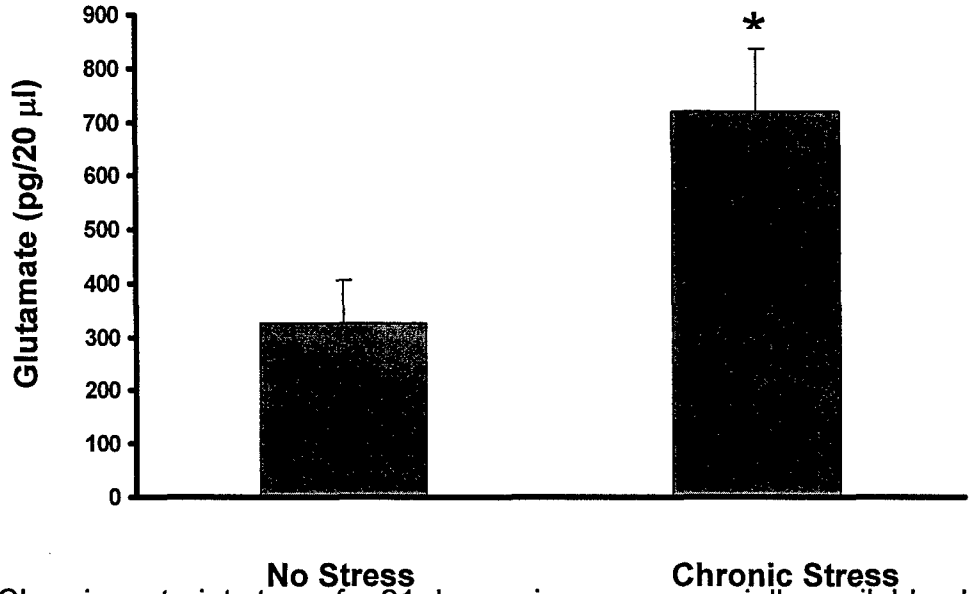


Fig. 2. Chronic restraint stress for 21 days using a commercially available plastic rat restrainer increases the basal extracellular concentrations of glutamate in the hippocampus (*p<0.05, T-test; n=8/group).

Discussion:
These findings illustrate that chronic stress can increase the extracellular concentrations of glutamate and perhaps account for the hippocampal remodeling and apical dendritic atrophy that has been observed by others (McEwen, 1997). The cause of the increase in extracellular glutamate is unclear however, it can be speculated that chronic elevations of glucocorticoids and calcium influx may cause the release of glutamate from hippocampal pyramidal cells. Another possibility is that chronic stress may oxidatively damage the glial glutamate transporter and diminish the uptake of glutamate and cause an accumulation of extracellular glutamate within the hippocampus.

Objective 3:
To examine the effect of methamphetamine on GABA release in the substantia nigra (SN) and the local regulation of GABA by DA as an index of the outflow activity of the basal ganglia.

Introduction
High extracellular concentrations of DA and the excitatory amino acid glutamate (GLU) have been implicated in mediating METH toxicity (Nash and Yamamoto, 1992). Systemic administration of METH increases both DA and GLU release. However, while local perfusions of METH directly into the striatum do produce an increase in DA release they do not produce an increase in GLU and do not produce long term depletions of striatal DA tissue content (Burrows et al., 2000).

This suggests that increases in both DA and GLU are necessary to produce neurotoxicity.

Although GLU appears to be significant in mediating METH toxicity, it is still unclear how METH increases GLU. Our hypothesis is that METH will increase extracellular GLU via the striatal outflow pathways, specifically the striatonigral efferents. We predicted that stimulation of the D1 receptors in the SN will increase GABA release in the SN.

Results: (See **Appendix 3**; Figure numbers below refer to the figures in the appendix)
High-dose METH significantly increased GAD65 mRNA levels by 35.6% and 29.7% in the ventral and dorsal neostriatum, respectively (Fig 1A and B), of rats killed 5 hrs after METH compared to saline-treated rats (Ventral neostriatum: METH: 0.1440± 0.007 and Saline: 0.106± 0.006 (Mean relative O.D ± SEM), METH vs Saline; T= 3.92, p<0.05; Dorsal neostriatum: METH: 0.135± 0.005 and Saline: 0.104±0.007 (Mean relative O.D ± SEM), METH vs Saline; T=3.35; p<0.05). There was no significant difference in METH-induced increases in GAD65mRNA expression between the dorsal and ventral regions of the neostriatum.

Figure 3 (Appendix 3) shows extracellular GABA concentrations in substantia nigra pars reticulata (SNr). METH + Vehicle (METH + VEH) treatment group had a significant increase in extracellular GABA concentrations compared to METH + SCH23390 (2-way ANOVA with repeated measures, SCH23390 simple main effect, $F_{(1,72)}$ = 5.17, p<0.05). There is no difference between Saline + Vehicle (Saline +VEH), Saline + SCH23390 and METH + SCH23390 treated rats. n=8-9 rats per group. The METH-induced increase in nigral extracellular GABA concentrations was D1 receptor dependent since intranigral perfusion of the D1 DA antagonist SCH23390 (10 μM) attenuated the METH-induced increase in GABA release in the SNr.

Figure 4 (Appendix 3) illustrates that METH decreased extracellular GABA concentrations in the ventromedial thalamus (VM). Intranigral perfusion of the GABA-A receptor antagonist, bicuculline (10 μM), blocked the METH-induced decrease in extracellular GABA in the VM. METH + Vehicle (METH+ VEH) group showed a significant decrease in extracellular GABA concentrations compared to METH+ BIC (2-way ANOVA with repeated measures, BIC main effect; $F_{(1,379)}$ = 15.34, p<0.05). There was no difference between Saline + Vehicle (SAL+VEH), Saline + BIC and METH+ BIC treated rats. n=9-10 rats per group.

Figure 5 (Appendix 3) shows that the METH-induced increase in striatal GLU was blocked by the intranigral perfusion of GABA-A receptor antagonist during the systemic administrations of METH . METH + Vehicle (METH+ VEH) group showed a significant increase in extracellular GLU concentrations compared to METH+ BIC (2-way ANOVA with repeated measures; BIC main effect, $F_{(1,339)}$ =

7.764, $p<0.05$). There was no difference between Saline + Vehicle (SAL +VEH), Saline + BIC and METH+ BIC treated rats. n=8-9 rats per group.

Importantly, Figures 6 and 7 (Appendix 3) show that the intranigral perfusion of a D1 antagonist (SCH23390) or a GABA-A antagonist (bicuculline), respectively, attenuated the striatal DA depletions when measured one week later.

Overall, these results show that METH (1) enhances D1-mediated striatonigral GABAergic transmission, that in turn (2) activates GABA-A receptors in the SNr leading to (3) a decrease in GABAergic nigrothalamic activity, (4) an increase in corticostriatal GLU release and (5) a consequent long-term depletion of striatal DA content.

Discussion:
Basal and stimulated GABA release in the SN is modulated by D1 receptors. Furthermore, neurotoxicity to DA terminals in the STR after METH is partially mediated by activation of D1 receptors and GABA release in the SN.
It can be postulated from the circuitry model illustrated in Appendix 3 (Fig. 8) that an increase of extracellular GABA in the SN will result in the activation of the thalamocortical projections and a subsequent increase in extracellular GLU release in the striatum.

The METH-induced increases in striatal GAD65 mRNA expression (Fig.1) and extracellular GABA within the SNr (Fig.3) appear to be mediated by DA within these respective brain regions. GAD65 mRNA expression was used as an index of GABAergic activity within the striatonigral pathway. To our knowledge, this is the first report of specific changes in striatal GAD65 mRNA expression after METH. Although we cannot conclude that increases in striatal GAD65 directly translate into increases in GABA release in the SNr, the increases in striatal GAD65 mRNA may reflect long-term changes in GABAergic activity within the striatonigral pathway after METH. GAD65 gene expression in striatonigral neurons is increased by D1 activation (Laprade and Soghomonian, 1995;Laprade and Soghomonian, 1997). Therefore, METH-induced striatal DA release (Stephans and Yamamoto, 1994) presumably activates striatal D1 receptors to increase GAD65 mRNA expression.

The increase in GABA after METH is likely mediated by D1 receptors within the SNr. These acute increases in SNr extracellular GABA were blocked by local perfusion of the D1 antagonist, SCH23390 into the SNr (Fig.3). Amphetamine increases DA release from dendrites of DAergic neurons in the SNr (Geffen et al., 1976;Heeringa and Abercrombie, 1995). The increase in extracellular DA can then activate D1 receptors present on striatonigral terminals (Porceddu et al., 1986);Altar and Hauser, 1987) to increase extracellular GABA (Aceves et al., 1995;Timmerman and Abercrombie, 1996; Matuszewich and Yamamoto, 1999). It is uncertain if SNr GABA measured in our study originates from striatonigral or pallidonigral terminals. Since D1 antagonism blocks METH-induced increases in extracellular GABA within the SNr and other findings showing that SNr D1 receptors are located primarily on striatonigral terminals, METH probably

activates the striatonigral GABAergic pathway via striatal GAD65 mRNA and a D1-mediated increase in GABA release from striatonigral terminals.

The major projection from the SNr is to the ventral medial nucleus of the thalamus (VM) (Somogyi et al., 1979;Bevan et al., 1994). GABA tonically inhibits GABAergic neurons in the SNr via GABA-A receptors (Rick and Lacey, 1994). Additionally, intranigral activation of GABA-A receptors located on GABAergic soma within the SNr that project to the motor thalamus decrease thalamic neuron firing (Deniau and Chevalier, 1985). Along these lines, the D1 stimulation increased extracellular GABA concentrations in the SNr and motor activity (Trevitt et al. 2002), the latter presumably mediated through SNr GABAergic neurons that innervate the VM (Faull and Carman, 1968;Beckstead et al., 1979). Therefore, our finding that METH acutely decreases extracellular GABA within the VM (Fig.5) can be explained by the inhibition of the nigrothalamic pathway resulting from increases in extracellular GABA in the SNr (Fig.3).

GABAergic neurons of the SNr innvervate and inhibit VM neurons (Di Chiara et al., 1979), whereas inhibition of SNr activity by intranigral application of GABA increases the activity of a large percentage of thalamocortical neurons (Deniau et la., 1985). Thus, the METH-induced increase in SNr GABA is probably associated with a decrease in extracellular GABA in the VM mediated by a decrease in impulse flow originating from the activation of SNr GABA-A receptors. This interpretation is supported by the finding that perfusion of the GABA-A antagonist, BIC, into the SNr blocked the METH-induced decrease in extracellular GABA in the VM (Fig.4).

The decreases in extracellular GABA within the VM after METH (Fig.5) can alter thalamocortical glutamatergic activity and subsequently, corticostriatal GLU transmission. Since VM glutamatergic neurons innervate the motor cortex (Moran et al., 1982), the METH-induced decrease in GABA in the VM may disinhibit the thalamocortical glutamatergic pathway and increase cortical activity. In fact, METH produces excitotoxicity in the motor cortex as evidenced by fluorojade immunoreactivity (Eisch et al., 1998) and a long-term decrease in NMDA receptor binding (Eisch et al., 1996). The acute increase in cortical extracellular GLU after METH (Burrows and Yamamoto, 2003) can presumably increase corticostriatal activity and explain the METH-induced increase in extracellular GLU (Nash and Yamamoto, 1992). Moreover, cortical ablation attenuates the METH-induced increases in extracellular striatal GLU (Burrows and Yamamoto, 2003) and suggests that activation of the corticostriatal glutamatergic pathway plays a role in the excitotoxicity to striatal DA terminals.

METH depletes striatal DA content when measured 7 days after drug treatment. A disruption of the METH-induced changes in the striatonigral or nigrothalamic pathways was posited to alter the acute METH-induced increases in extracellular GLU in the striatum and consequently, the long-term depletion of striatal DA content. In fact, D1 antagonism attenuated both the acute METH-induced increase in extracellular GABA in the SNr (Fig.3) and the long-term depletions of striatal DA tissue content measured 7 days later (Fig.6). In addition to the

blockade of the METH-induced decreases in extracellular GABA in the VM (Fig. 4) by BIC perfusion in the SNr, the acute increase in extracellular GLU (Fig. 5) and the subsequent long-term depletion of striatal DA was also blocked on the side ipsilateral to the local perfusion of BIC (Fig. 7). In contrast, intranigral perfusion of SCH 23390 only attenuated but did not completely block the METH-induced DA depletions in striatum (Fig.6). One explanation is that BIC more directly and effectively alters the nigrothalamic pathway via convergent inputs from the globus pallidus and striatum onto GABA-A receptors, whereas SCH23390 alters D1-mediated GABA release only from striatonigral terminals to affect nigrothalamic GABAergic transmission.

In conclusion, long-term striatal DA depletions produced by METH are in part, due to activation of the basal ganglia outflow pathway. Figure 8 illustrates that METH (Fig. 8B) activates the direct striatonigral GABAergic pathway via increased DA release in the striatum and SNr and activation of D1 receptors in the SNr to inhibit nigrothalamic GABA transmission, a subsequent disinhibition of thalamocortical glutamate release, and an eventual increase corticostriatal GLU.

Objective 4:
To characterize the input from the subthalamic nucleus to the substantia nigra.

Details of these experiments are described in **Appendix 4. Appendix 4** is a manuscript that will be submitted for publication. Figure numbers refer to the figures in the Appendix.

Introduction:
The goal of these experiments was to investigate the regulation of the glutamatergic projection from the subthalamic nucleus to the substantia nigra utilizing the technique of dual-probe microdialysis in the awake behaving rat. Reverse dialysis of the selective D_1 antagonist SCH-23390 or the selective D_2 antagonist raclopride into the SN was used to assess the differential contributions of these two receptor subtypes on glutamate release in the substantia nigra during carbachol-stimulation of the STN. Carbachol is a known muscarinic agonist and has been used previously to stimulate the STN. Furthermore, the contribution of GABA on glutamate release induced by STN stimulation was assessed by perfusing the selective $GABA_A$ antagonist bicuculline into the SN during stimulation of the STN. It was posited that the administration of cholinergic receptor agonist, carbachol into the subthalamic nucleus will stimulate glutamate and dopamine release in the substantia nigra. It was further hypothesized that the increase in extracellular dopamine within the substantia nigra negatively modulates the stimulated release of glutamate from the subthalamic terminals via the activation of D_2 receptors.

Results:
Reverse dialysis of the muscarinic cholinergic receptor agonist carbachol into the subthalamic nucleus transiently increased the extracellular concentrations of glutamate in the substantia nigra (Fig. 1; *$p<0.05$, n=7-8/group) and was

subsequently followed by an increase in extracellular dopamine (Fig. 4; *$p<0.05$, n=5-7/group).

Carbachol-stimulated glutamate release was enhanced and prolonged by perfusion of the selective D_2 dopamine receptor antagonist raclopride into the substantia nigra (Fig. 2; *$p<0.05$, n=5-8/group). In contrast, the D_1 dopamine receptor antagonist SCH-23390 (Fig. 2, n=5) and the $GABA_A$ receptor antagonist bicuculline (Fig. 3, n=5-8/group) did not affect carbachol-stimulated glutamate release. Statistical significance was determined by a two-way ANOVA with repeated measures coupled with Bonferroni's post hoc test ($p<0.05$).

Discussion

These data indicate that stimulation of muscarinic receptors by carbachol in the subthalamic nucleus results in an increase in subthalamic activity to increase glutamate release in the substantia nigra. We plan to examine the regulation of the subthalamonigral pathway by metabotropic and dopaminergic receptors in the substantia nigra. The rapid return of glutamate towards basal values despite the continued stimulation by carbachol may indicate that there is a negative feedback control of stimulated glutamate release.

The stimulatory effects of carbachol in the STN on glutamate release in the substantia nigra are most likely mediated via the activation of muscarinic M_3 cholinergic receptors present in the STN (Flores et al., 1996) and not nicotinic cholinergic receptors since the latter do not contribute significantly to the activity of subthalamic neurons (Feger et al. 1979).

Although reverse dialysis of carbachol in the STN increased the extracellular concentrations of glutamate in the SN, the increase was transient and returned to basal values despite the continued perfusion of carbachol. One possible explanation is that cholinergic receptor desensitization in the STN may have contributed to the lack of a sustained increase in extracellular glutamate within the SN. However, this possibility is unlikely since local pressure application of high concentrations (10 mM) of carbachol did not reduce the firing of subthalamic neurons (Falkenburger et al. 2001). Another possibility that was considered and addressed in the current study is that the subthalamonigral glutamatergic projection is tightly regulated by an inhibitory dopaminergic feedback mechanism initiated within the substantia nigra at the glutamatergic terminals.

The antagonism of D_2 receptors within the SN enhances appears to prolong the increase in glutamate produced by carbachol stimulation of the STN. Therefore, the increase in dopamine observed within the SN during carbachol stimulation of the STN (Fig. 4) may limit the increase in glutamate via activation of D_2 receptors localized presumably on asymmetric excitatory terminals (Pickel et al. 2002).

In contrast, perfusion of the D_1 receptor antagonist SCH-23390 into the SN did not enhance or prolong the transient increase in extracellular glutamate produced by carbachol. These findings do not suggest a role for D_1 receptors in the regulation of glutamate release from STN terminals as proposed previously

(Rosales et al. 1997). The major difference between the current findings and those of Rosales et al. (1997) is that they evaluated the role of D_1 receptors on basal glutamate concentrations compared to the present study that examined the role of D_1 receptors in stimulated glutamate release. Therefore, D_1 receptors may play a role in the regulation of basal glutamate concentrations but not under stimulated conditions when the STN is activated.

Another possibility that was considered is that the regulation of subthalamonigral glutamate transmission can be mediated by the inhibitory neurotransmitter GABA through the direct activation of GABA heteroreceptors on the STN axon terminals or through the modulation of dopamine concentrations in the SN. GABAergic input into the SN originates from the striatum and pallidum (Bolam & Smith 1990; Smith & Bolam 1990) and from intrinsic GABAergic interneurons (Grofova et al. 1982). GABA is critically involved in the regulation of DA cell activity by tonically suppressing the excitatory inputs (Kitai et al. 1999). This effect is mediated primarily via $GABA_A$ receptor activation localized on DA cell bodies (Tepper et al. 1995; Paladini et al. 1999). In the present study, perfusion of the $GABA_A$ receptor antagonist bicuculline into the SN during carbachol stimulation of the STN had no effect on glutamate release produced by STN stimulation. This indicates that GABA does not appear to directly regulate glutamate release from STN axon terminals and further highlights the importance of dopamine release (Fig. 4) and the D2 receptor in regulating glutamate release in the SN during STN stimulation.

Consistent with the goal of determining the role of glutamate excitotoxicity in the damage produced by METH to the basal ganglia, we examined the effect of METH on extracellular glutamate in the substantia nigra in Objective 6

Objective 5:
To examine the acute changes in the extracellular concentrations of glutamate in the substantia nigra after METH in the presence or absence of raclopride perfusion into the SN and to examine the long-term effects of these manipulations on dopamine tissue content of the SN.

Introduction:
The perfusion of raclopride into the SN during the infusion of carbachol into the STN prolonged the increase in glutamate with the SN. To examine the interaction between the effects of systemic administration of METH and D2 blockade, we measured changes in extracellular glutamate as measured by microdialysis. It is hypothesized that METH should produce a long-term decrease in dopamine content in the striatum due to the excitotoxic effects of glutamate. Furthermore, repeated treatment of the longer acting D2 antagonist, haloperidol for 5 days after the administration of METH will produce a decrease in dopamine content in the striatum and in the substantia nigra.

Results:
The systemic administration of METH and raclopride increased the extracellular concentrations of glutamate in the SN (Fig. 3 below). METH alone did not produce a significant increase in glutamate.

FIGURE 3

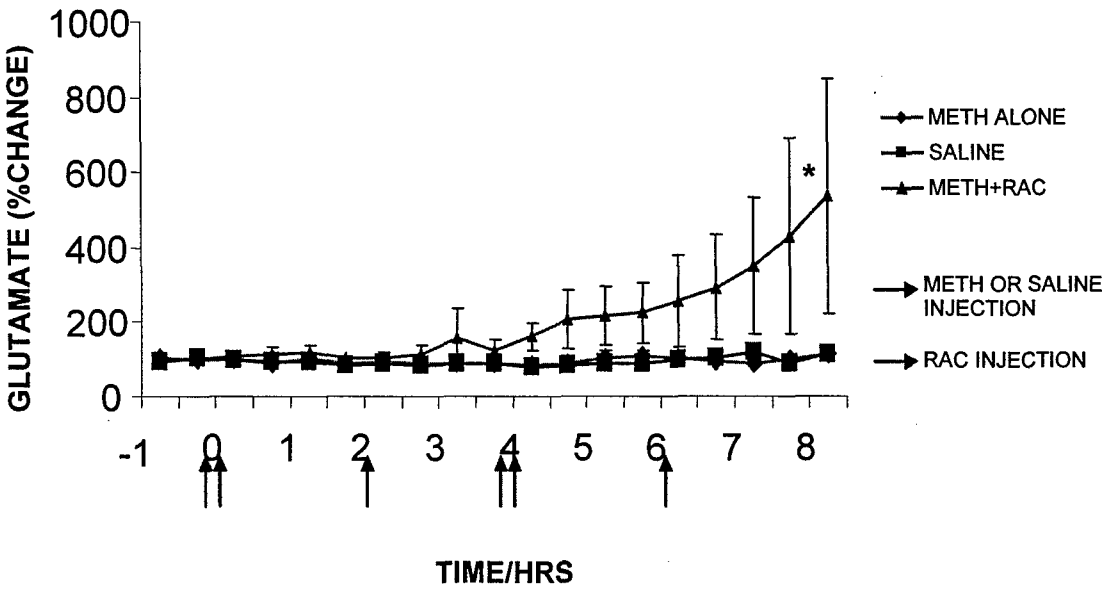


Fig. 3 Systemic METH + RACLOPRIDE (RAC) perfusion of the SN increased the extracellular concentrations of glutamate in the substantia nigra (SN). *$p<0.05$ compared to METH alone (ANOVA with repeated measures, overall main effect of raclopride). Arrows indicate times of METH/saline and raclopride (RAC) injections. N=6-8/group

METH alone or the subchronic administration of the D2 antagonist, haloperidol (0.5 mg/kg, ip) for 5 days after the administration of METH produced a depletion of dopamine content in the striatum (Fig. 4). More importantly, the post-METH administration of haloperidol produces a decrease in dopamine content in the substantia nigra 7 days after the METH treatment (Fig. 5).

Figure 4

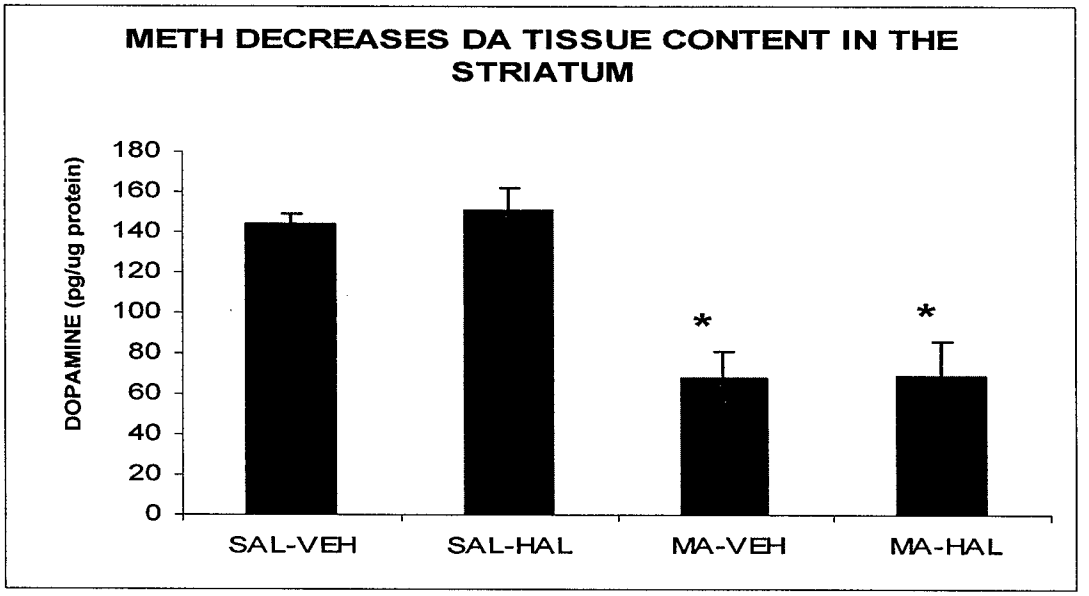


Figure 4. Effect of METH and/or haloperidol on dopamine content in the striatum *p<0.05 compared to Sal-VEH and SAL-HAL, tukey post hoc test. N=8-15/group

MA=METH; HAL=haloperidol; SAL=Saline; VEH=vehicle

Figure 5

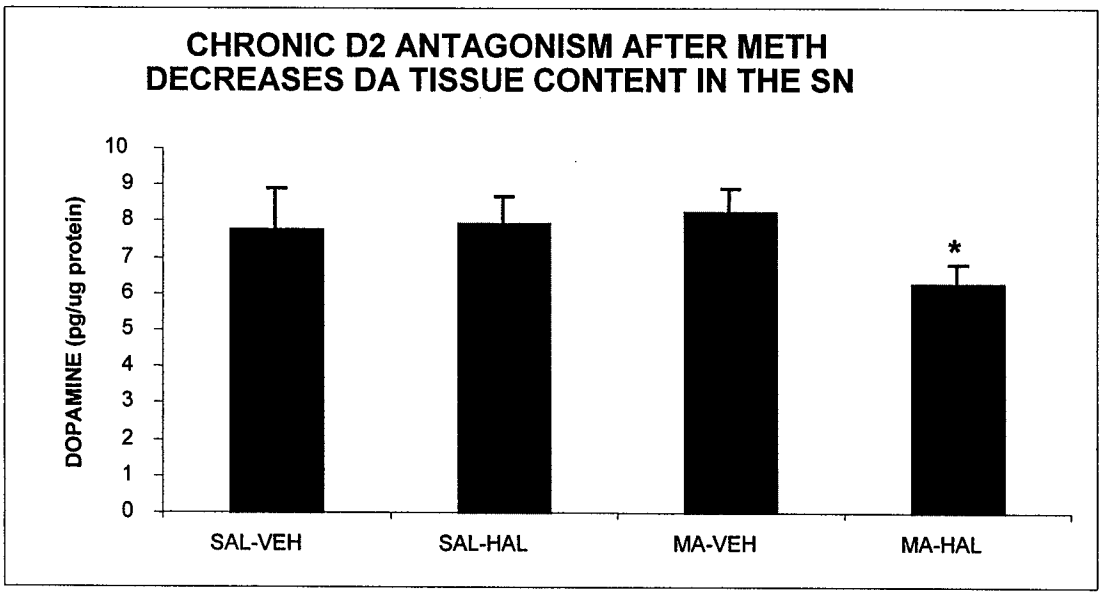
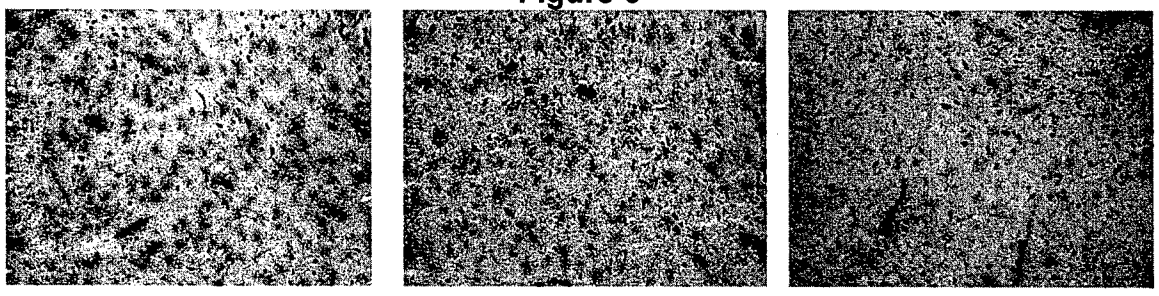


Figure 5. Effect of METH and/or haloperidol on dopamine content in the substantia nigra *p<0.05 compared to MA-VEH, Tukey post hoc test. N=8-15/group

To examine if methamphetamine alone or in combination with haloperidol decreased cell number in the substantia nigra, separate groups of rats were treated as described above and NeuN staining of neuronal nuclei was examined in the substantia nigra.

The combined treatment of methamphetamine (MA) and haloperidol (HAL) produced a decrease in neuron number in the substantia nigra pars reticulata (SNr). Figure 6 illustrates representative histological sections of NeuN staining. SAL-VEH = saline followed by repeated tartaric acid vehicle, MA-VEH = methamphetamine (10 mg/kg ip q 2 hr X 4) followed by repeated tartaric acid vehicle, MA-HAL= methamphetamine followed by repeated haloperidol (0.5 mg/kg ip once daily for 5 days).

Figure 6



SAL-VEH **MA-VEH** **MA-HAL**

Figure 7

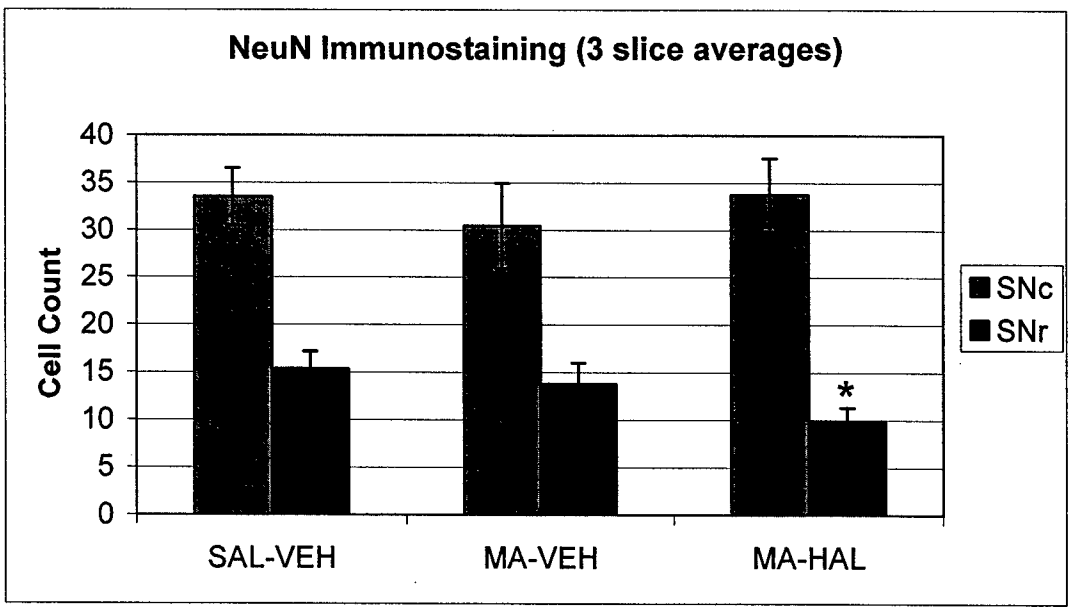


Fig. 7 Cell counts in the substantia nigra pars compacta (SNc) and substantia nigra pars reticulata (SNr). MA = methamphetamine, SAL= Saline, VEH = tartaric acid vehicle, *p<0.05 compared to MA-VEH; n=9-10/group

To confirm that this regimen produced damage to dopamine terminals in the striatum, dopamine transporter (DAT) protein immunoreactivity was examined (Fig. 8). In addition, dendritic spectrin was also measured as an indicator of cell morphology changes (Fig. 9). DAT and dendritic spectrin levels were significantly depleted in the MA-VEH and MA-HAL groups compared to controls (*$p<0.05$; post hoc Tukey test) but the MA-HAL group was not significantly different from MA-VEH.

Figure 8

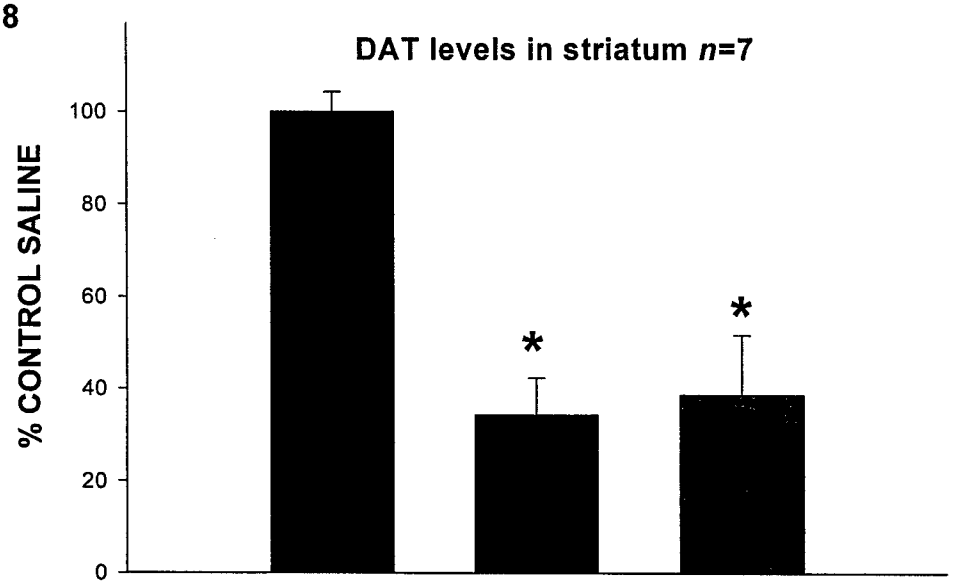
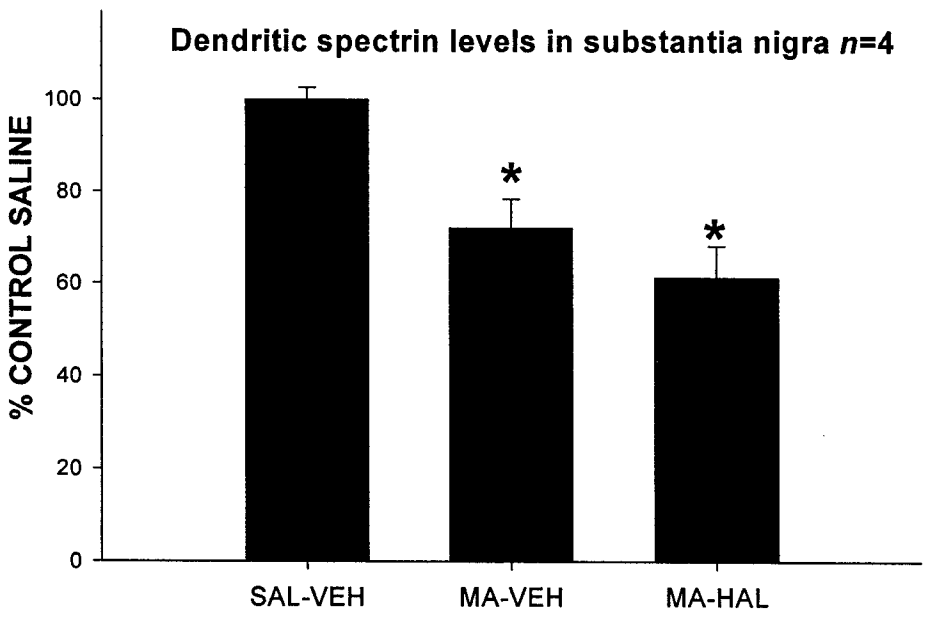


Figure 9



Discussion:
These data indicate that METH by itself decreases DAT immunoreactivity in the striatum (Fig. 8) but does not affect the SN as manifested by the lack of a long-term depletion of SN dopamine content (Fig. 9). However, it may have toxic effects to cell bodies with regard to dendritic proteolysis. Thus, it appears that METH alone causes dendritic retraction or proteolysis of cell bodies in the SN. The phenotype of those cell bodies and their exact location within the subregions of the substantia nigra are unknown at present but they may be either dopaminergic or GABAergic cell bodies. Nevertheless, dendritic retraction/proteolysis can occur in the absence of loss of cell bodies produced by METH alone as indicated by no changes in NeuN staining of neuronal nuclei. The absence of cell body loss after METH alone may be explained by the lack of increase in extracellular glutamate in the SN during the administration of METH (Fig. 3).

These findings, in combination with the studies in Objective 3 showing that the D1-regulated direct output pathway of the basal ganglia is affected by METH, illustrate that METH primarily affects the D1 direct pathway via GABA and not the indirect pathway mediated by D2 receptors and glutamate from the subthalamic nucleus. However, if glutamate release from the subthalamonigral terminals is disinhibited by the antagonism of D2 receptors in the SN, extracellular glutamate concentrations are increased (Fig. 3) which in turn, may produce damage to dopamine cell bodies in the SNr (Figs. 6 and 7). These data have significant implications for METH toxicity and the potential for the development of motor disorders, particular since D2 antagonists such as haloperidol are used acutely for the emergency treatment of METH-induced psychosis. It remains to be seen if chronic stress can activate the subthalamonigral glutamatergic pathway and be additive with the effects of METH to damage cell bodies within the SN.

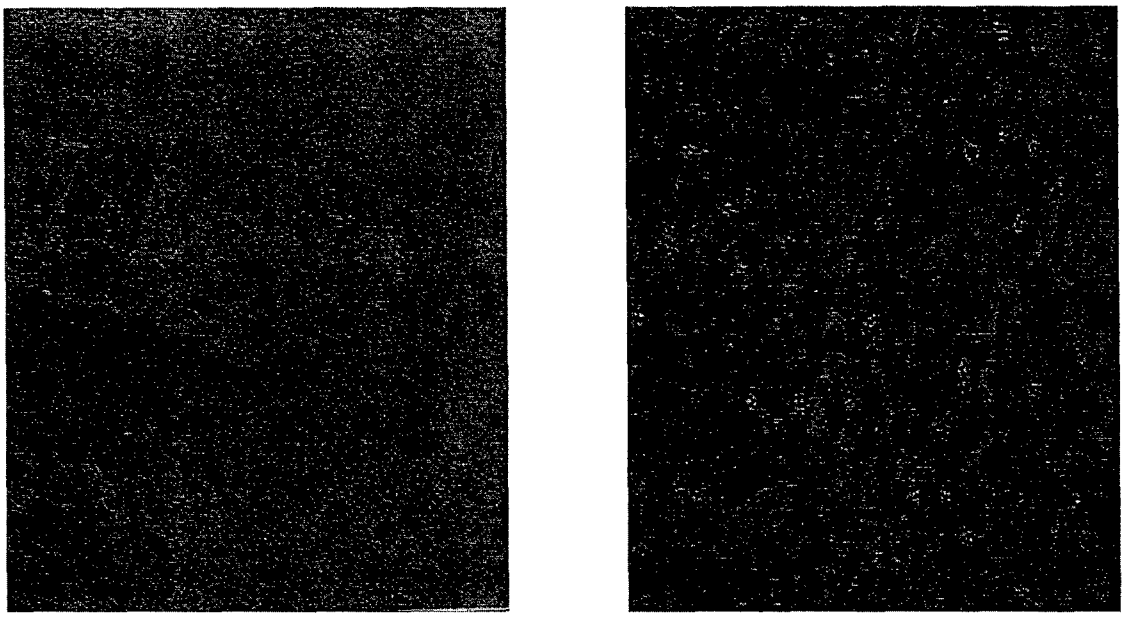
Objective 5:
To examine the effect of methamphetamine on oxidative damage in the striatum as measured by protein nitration.

As described in the Progress Report last year, methamphetamine increased the formation of nitrotyrosine in the striatum, as measured by HPLC analysis of acid hydrolyzed protein. To verify this finding with another method, we employed the immunohistochemical detection of an anti-nitrotyrosine antibody 24 hrs after the administration of methamphetamine.
In addition, to examine the effects of prior exposure to chronic stress on METH-induced nitrosative oxidative stress, we examined nitrotyrosine concentrations by HPLC analysis of hydrolyzed protein.

Results:
As can be observed in Figure 10 below shows a representative histological slice from a rat from each treatment group). There was increased immunohistochemical staining observed in the striatum of methamphetamine

treated rats compared to saline (126.4 +0.2 vs, 22.1+0.5 arbitrary optical density units, $p<0.05$; T-test).
Figure 11 illustrates that prior exposure to chronic unpredictable stress enhanced the formation of nitrotyrosine produced by METH alone in non-stressed rats. These are new data since the last submission of the Final Report.

Fig. 10



Saline **Methamphetamine**

Figure 11

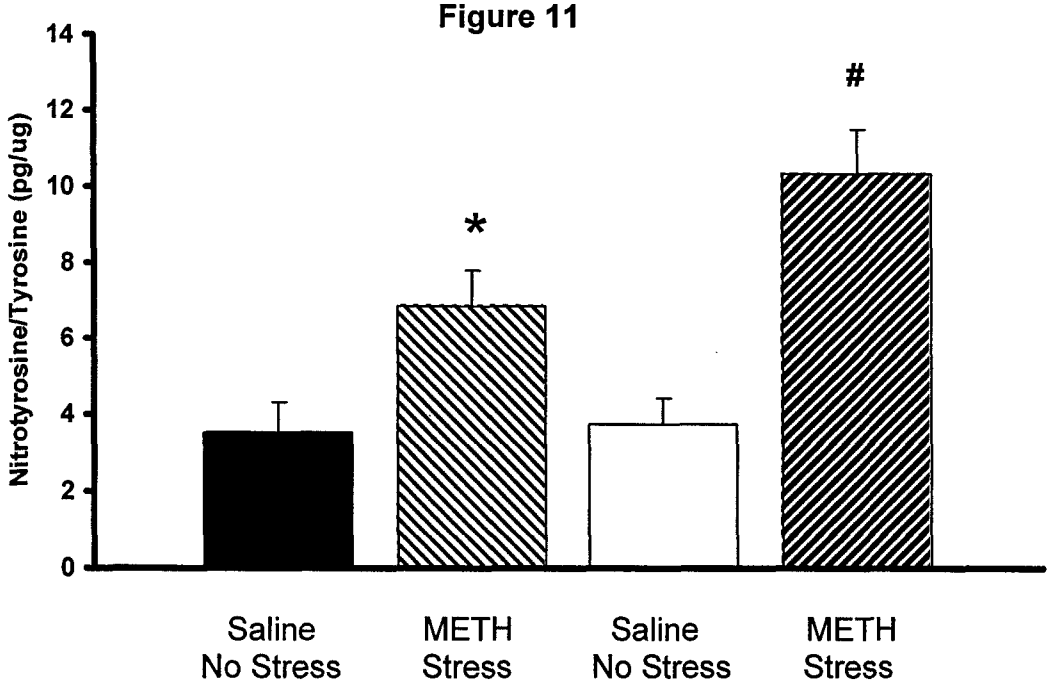


Figure 11. Effect of METH and/or Stress of nitrotyrosine concentrations in the striatum. *$p<0.05$ compared to Saline-No stress; #$p<0.05$ compared to METH-Stress

Discussion:
The results indicate that methamphetamine produces evidence of oxidative stress in the striatum as indicated by the nitration of protein in the form of nitrotyrosine. These data are consistent with our previously published studies showing the METH increases the extracellular concentrations of glutamate and dopamine that in turn, could lead to the formation of glutamate-induced nitric oxide and dopamine-derived production of hydrogen peroxide. Subsequently, nitric oxide and hydrogen peroxide can combine to form the highly reactive peroxynitrite and the subsequent nitration of protein as evidenced by nitrotyrosine. Moreover, prior exposure to chronic stress enhanced the nitration of tyrosine residues and indicates that stress enhances the oxidative damage produced by METH presumably through the enhanced release of glutamate.

Objective 6:
To examine the effects of METH on the activity and protein content of electron transport chain complexes of the mitochondria.

Introduction:
Although we have shown that glutamate and oxidative stress appear to mediate the damage to dopamine terminals, the exact targets of this damage is not known. Since we have also shown that METH produces a bioenergetic compromise, we hypothesized that METH inhibits mitochondrial function, increasing the free radical burden and decreasing neuronal energy supplies. Moreover, previous studies have shown that either complex II inhibition or METH results in loss of markers for dopamine terminals in the striatum without affecting dopamine cell bodies in the substantia nigra (Zeevalk et al. 1997; Blum et al., 2004; Ricaurte et al., 1982). Therefore, the purpose of the present studies was to determine if METH administration selectively inhibits complex II of the ETC in rats.

Results: Details are found in **Appendix 5** which is a manuscript accepted for publication and in press in the *Journal of Neurochemistry* (Brown, J,M. Quinton, M.S. and Yamamoto, B.K), 2005. **Figure numbers in the text below refer to the corresponding numbers in Appendix 5.**

High-dose METH administration (10 mg/kg every 2 h × 4) rapidly (within 1 h) decreased complex II (succinate dehydrogenase) activity by ~20-30%. In addition, decreased activity of complex II-III, but not complex I-III, of the mitochondrial ETC was also observed 24 h after METH (Fig. 1) and was specific for the striatum and did not occur in the hippocampus (Fig. 2). The inhibition of complex II in the striatum was not due to direct inhibition by METH (Fig. 3) or METH-induced hyperthermia (Fig. 1). METH-induced decreases in complex II-III were prevented by MK-801 (Fig. 5) and the peroxynitrite scavenger, Fe-TPPS (Fig. 7).

To determine if the decrease in Complex II activity was due to a loss of the enzyme protein, the immunoreactivities of catalytic 70 kD subunit and the 30 kD subunit were measured. METH produced a decrease in the immunoreactivity of both the 70 kD and 30 kD subunits of Complex II (Fig. 12). These data are *not* in the appended manuscript.

Figure 12

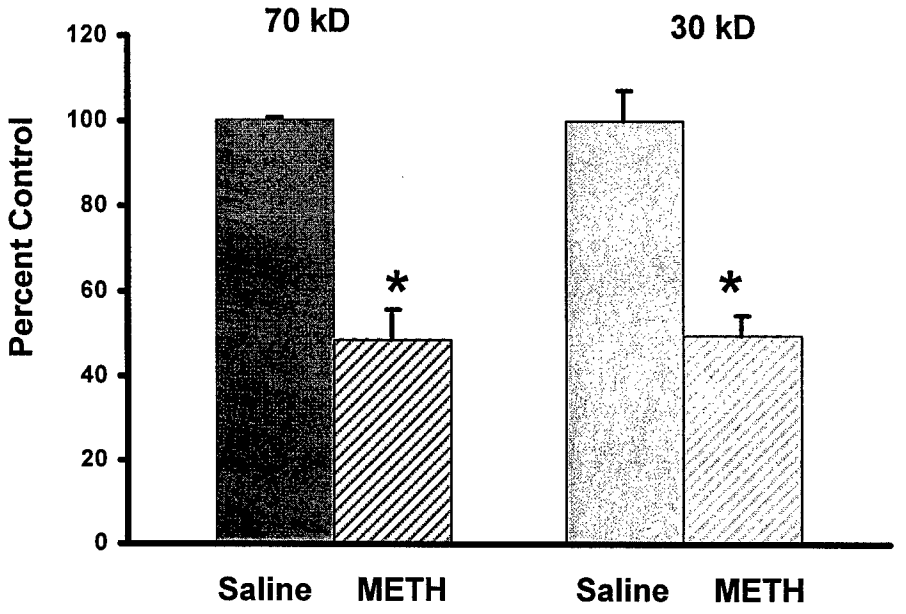


Fig. 12. Effects of METH on Complex II protein subunits.24 hrs after METH (10 mg/kg ip q 2 hr X 4). *p<0.05 compare to respective saline controls; T-test.

Discussion:

High-dose METH administration rapidly and selectively inhibited the activity of complex II in the mitochondrial ETC (Figure 1). This inhibition of complex II activity was specific for striatal, but not hippocampal brain regions (Figure 2) and is unrelated to METH-induced hyperthermia (Figure 1) or residual METH in the mitochondrial preparation (Figure 3).

Several experiments were conducted to elucidate the mechanism mediating the decrease in complex II activity. The observed effects are not due to a direct effect of METH, since incubation of mitochondria with concentrations of METH that are typically achieved in suspensions of striatal tissue (~48nM) after a systemic injection regimen (Riddle et al., 2002) had no effect on complex II-III or I-III activity. Only millimolar concentrations of METH produced a non-selective inhibition of complexes I-III (data not shown) and II-III (Figure 3). In addition, the decrease in complex II-III was not associated with METH-induced hyperthermia since prevention of hyperthermia did not reverse the decrease in complex II-III activity (Figure 1). Previous studies showed that prevention of hyperthermia is neuroprotective against METH-induced dopaminergic deficits (Albers and

Sonsalla, 1995). However, it was concluded in these studies that hyperthermia contributes to, but is not solely responsible for, METH-induced deficits (Albers and Sonsalla, 1995). Thus, the decrease in complex II-III may mediate a hyperthermia-independent component of METH toxicity to dopaminergic elements. Alternatively, hyperthermia may enhance the toxic effects of mitochondrial inhibition but does not contribute directly to mitochondrial inhibition.

Although the exact mechanism of this decrease in complex II remains to be determined, METH-induced increases in striatal glutamate appear to contribute. Specifically, administration of an NMDA receptor antagonist MK-801, even injected *after* the administration of METH, prevented METH-induced decreases in activity of complex II-III (figure 5). Dabbeni-Sala et al. (2001) demonstrated that incubation with a glutamate receptor agonist causes a selective loss of complex II activity. These effects were prevented by a glutamate receptor antagonist and antioxidant treatment (i.e. melatonin or GSH). METH produces a delayed increase in striatal glutamate (Nash et al., 1992), which can, in turn, activate glutamatergic ionotropic receptors and damage dopamine terminals in the striatum. This increased stimulation of glutamate receptors may initiate a feed-forward mechanism that occurs predominately *after* the METH administration regimen and results in inhibition of complex II. The finding that administration of MK-801 *after* METH can prevent the decrease in complex II activity is consistent with the delayed rise in glutamate (Nash and Yamamoto, 1992) and suggests that late occurring excitotoxic events to complex II can still be blocked pharmacologically. These data may have significant implications for the treatment of METH overdose.

Glutamate-mediated receptor activation has been linked with NOS activation, protein nitration and peroxynitrite formation in the striatum (Ayata et al., 1997). Numerous studies have implicated NOS and peroxynitrite as key players in protein nitration and METH-induced deficits (Imam et al., 2001b). Results presented in figure 5 demonstrate that complex II-III activity showed a greater sensitivity to inactivation by ONOO- when compared to complex I-III activity. A greater sensitivity of complex II to ONOO- has been shown previously by others. For example, Murray et al. (2003) demonstrated that complex I and II were equally inhibited by incubation with 800 μM ONOO but complex II showed a greater inhibition at higher concentrations (1.6mM ONOO-). These results parallel the finding in the present study (Figure 6). In addition, results from Cassina and Radi (1996) demonstrated a preferential inhibition of complex II when compared to complex I by peroxynitrite. It should be noted that previous studies have demonstrated inhibition of complex I by reactivity nitrogen species (see Murray et al. (2003)). At the doses on ONOO- tested a trend, albeit not significant, decrease in complex I-III activity was noted. Alternatively, METH-induced glutamate release could also lead to inhibition of complex II through the increased intracellular Ca^{2+} concentrations (Kushnareva et al., 2005; Ward et al., 2005) and generation of reactive oxygen species (Kahlert et al., 2005). Furthermore, Ayata et al. (1997) showed that NMDA receptor activation in nNOS knockout mice could still produce damage to the striatum, despite the absence of protein nitration. Although the size of the lesions was reduced by 50% when

compared to the wild-type animals that exhibited extensive protein nitration, these results suggest that glutamate could also lead to neuronal toxicity that is independent of NOS activation. In some experiments presented in the current study a non-significant trend for decreases in complex I-III was noted. Therefore the potential exists for some inhibition of complex I-III activity but this inhibition appears less than that of complex II.

The link between peroxynitrite formation and METH-induced inhibition of complex II-III activity and METH was further supported by administration of the peroxynitrite scavenger Fe-TPPS. When administered 12 hr before the first and 5 min prior to the third METH administration, this agent reversed the METH-induced decrease in complex II-III activity (Figure 6). Interestingly, Fe-TPPS has also been shown to prevent the dopaminergic deficits induced by high-dose METH administration (Imam et al., 2001b), further supporting the link between METH, complex II inhibition, peroxynitrite, and long-term dopaminergic deficits.

These data indicate that METH produces a selective and rapid inactivation of Complex II of the mitochondria. This effect is not due to a direct effect of METH since only at mM concentrations of METH was there a decrease in enzyme activity when METH was directly incubated with the mitochondrial preparation, at least 100 times higher than what is achieved in the brain following a neurotoxic regimen with repeated systemic administrations. Moreover, the decrease was not due to the hyperthermic effects of METH since cooling the rats had no effect on the decrease in activity. The decrease in Complex II enzyme activity is most likely due to a degradation of the enzyme as evidenced by the decrease in protein subunit immunoreactivity.and is mediated by glutamate and the NMDA receptor and the formation of peroxynitrite.

Unlike other ETC complexes, little is known about the regulation of complex II by pharmacological agents. Although agents such as malonate directly inhibit complex II activity, the present study demonstrates that other pharmacological agents such as METH can indirectly, but selectively inhibit this complex through glutamate-, peroxynitrite-mediated mechanisms. This rapid and persistent loss of complex II activity produced by METH may have long-term consequences on neuronal function. These studies provide the first direct evidence that high-dose METH rapidly decrease complex II activity via glutamate-receptor, peroxynitrite-mediated mechanisms. This inhibition may represent the initial catalytic event that occurs predominately after and not during METH administration and contributes to METH-induced toxicities and perhaps other neurodegenerative disorders.

Objective 7:
To examine the excitotoxic effects of methamphetamine as measured by calpain-mediated spectrin proteolysis.

Introduction: Although we have shown that METH produces a delayed and sustained rise in glutamate, no studies have shown if METH produces structural evidence of excitotoxicity or have identified the receptors that mediate this toxicity directly, independent of alterations in METH-induced hyperthermia. These experiments investigated if METH can cause excitotoxicity as evidenced by cytoskeletal protein breakdown in a glutamate receptor-dependent manner. Spectrin breakdown products were measured by western blot analysis.

Results: The data are illustrated in described in **Appendix 6**. **Appendix 6** is a manuscript that has been submitted for publication to the *Journal of Neurochemistry*. Figure numbers in the text below refer to the Figure numbers in the manuscript.

METH increased calpain-mediated spectrin proteolysis in the rat striatum 5 and 7 days after METH administration (Fig. 1A) without affecting caspase-3 dependent spectrin breakdown (Fig. 1B) This effect was completely blocked with the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist GYKI 52466 (Fig. 4A) but not the NMDA receptor antagonist MK-801 (Fig. 6A). However, AMPA or NMDA receptor antagonism did not attenuate the METH-induced depletions of the dopamine transporter (DAT) (Figs. 4B and 6B, respectively).

Discussion:
The results suggest that METH-induced increases in extracellular glutamate (Nash and Yamamoto 1992) selectively activate AMPA receptors on DA terminals in the striatum to produce calpain-mediated spectrin proteolysis. However, the mechanisms underlying glutamate-mediated spectrin proteolysis appear to be independent of the mechanisms by which METH decreases DAT protein in the striatum.

The increase in calpain-mediated spectrin proteolysis appeared at both 5 and 7 days but not at 3 days after METH administration. Furthermore, the increase at 7 days was significantly less than the spectrin proteolysis observed at 5 days. This time course of spectrin proteolysis is in accordance with the time course of silver staining in the striatum after a multiple dosing METH regimen (Ricaurte et al., 1982). The late onset of spectrin degradation could be explained through the delayed yet sustained elevation of glutamate in the striatum during a neurotoxic METH regimen (Nash and Yamamoto 1992; Abekawa et al. 1994) and the time needed for the accumulation and detection of SBPs. Thus, persistent stimulation of glutamate receptors may be necessary to sufficiently increase the intracellular concentrations of calcium and calpain activity to degrade the terminal and permit the detection of accumulated SBPs.

The decline in immunoreactivity for the 145 kDa SBP at 7 days can be explained by the removal of spectrin fragments from the CNS following terminal degradation. It also has been shown that SBPs are cleared from the CNS and transported to the cerebrospinal fluid after global ischemia (Pike et al. 2001; Pike et al. 2004). Other studies have shown that spectrin proteolysis reaches a maximum level after 3 days following an ischemic episode or traumatic brain injury conditions that then declines over time (Beer et al. 2000; Zhang et al. 2002).

The present results showing an increase in the 145 kDa SBP over time after METH without a change in the 120 kDa SBP suggest that METH-induced spectrin proteolysis is mediated only through calpain activation and not through an increase in caspase-3 activity.

Although GYKI 52466 attenuates METH-induced spectrin proteolysis, it is unclear if the protective effect of the AMPA antagonist is through a direct blockade of AMPA receptors located on DA terminals or through an indirect decrease in the extracellular concentrations of glutamate. Microdialysis experiments have shown that GYKI 52466 does attenuate the rise in striatal glutamate during ischemia (Arvin et al. 1994). It is possible that GYKI 52466 decreases the METH-induced elevations of extracellular glutamate to ultimately attenuate spectrin proteolysis. While the present findings are supportive of the conclusion that GYKI 52466 may be acting directly at AMPA receptors located on DA terminals, future studies investigating if AMPA antagonism affects METH-induced increases in extracellular glutamate are warranted.
Microdialysis studies have indicated that AMPA receptors mediate DA release in the striatum (Ohta et al. 1994; Sakai et al. 1997; Segovia et al. 1997; Hernandez et al. 2003). These AMPA receptors appear to lack the GluR2 subunit (Betarbet and Greenamyre 1999; Lai et al. 2003) and demonstrate calcium permeability (Hollmann et al. 1991). Therefore, a massive influx of calcium could activate calpain within the DA terminal and thereby degrade the cytoskeleton through proteolysis of spectrin. In contrast, there is a paucity of evidence for NMDA receptor expression on DA terminals in the striatum (Keefe et al. 1993; Dunah et al. 2000; Lai et al. 2003). Our finding that MK-801 was not effective at decreasing METH-induced spectrin proteolysis when rats were maintained at hyperthermic temperatures is consistent with those studies

It is interesting to note that neither glutamate antagonist attenuated the METH-induced DAT depletions 5 days after the neurotoxic regimen. There are two possible explanations for the dissociation between the inability of the antagonist to attenuate the decrease in DAT protein while blocking the increase in spectrin proteolysis: 1) The mechanisms underlying spectrin proteolysis and DAT depletions could be independent of one another yet both effects are localized to DA terminals in the striatum or 2) spectrin proteolysis is not localized to DA terminals but to some other neuronal subtype in the striatum. The first possibility could be explained by the fact that while both oxidative stress and glutamate excitotoxicity have been implicated in METH-induced damage to DA terminals, (Wagner et al. 1985; Yamamoto and Zhu 1998; LaVoie and Hastings 1999;

Harold et al. 2000; Itzhak et al. 2000), excitotoxicity could occur independent of decreases in DAT protein produced by oxidative stress. Likewise, oxidative stress could target DAT compared to the excitotoxic effects to the cytoskeleton. Thus, even in the face of diminished cytoskeletal proteolysis with an AMPA antagonist, oxidative damage to DAT could still persist.

A second possibility is that spectrin proteolysis does not occur in DA terminals. A likely target is the 5-HT terminal. Striatal 5-HT terminals are damaged by systemic METH administration and 5-HT is released from these neurons following elevations of extracellular glutamate or the perfusion of NMDA into the striatum (Hotchkiss et al. 1979; Bakhit et al. 1981; Schmidt and Gibb 1985; Green et al. 1992; Ohta et al. 1994; Abellan et al. 2000). It is possible that spectrin proteolysis and loss of serotonergic terminals mediated through glutamatergic mechanisms is also correlated 5 days after a neurotoxic METH regimen. If glutamate receptor antagonism attenuates 5-HT toxicity in a temperature-independent manner, the mechanisms that lead to damage of 5-HT terminals in the striatum could be different from those mediating damage to DA terminals. This potential outcome may indicate a differential susceptibility of DA versus 5-HT neurons to glutamate-mediated excitotoxicity.

In summary, this is the first evidence that a neurotoxic METH regimen can produce structural damage to neurons in an excitotoxic manner that involves AMPA receptors and calpain activation. In contrast, AMPA or NMDA receptors do not appear to directly mediate the decreases in striatal DAT protein produced by METH. Therefore, the mechanisms underlying membrane degradation and damage to the dopamine transporter appear to be independent of one another. Considering that calpain activation has been implicated in Parkinson's disease, Huntington's disease and Alzheimer's disease, future studies that elucidate the specific neuronal phenotype undergoing METH-induced spectrin proteolysis are warranted.

Objective 8:
To examine the effects of subchronic low dose methamphetamine (METH) administration on regional changes in DAT and NET immunoreactivity and function during early withdrawal.

The effects of subchronic METH on stress responsivity with respect to DA release in the NA SHELL and CORE during acute restraint stress were examined to evaluate if low doses of METH produce any evidence of toxicity or changes in stress reactivity. These studies are an extension of the aims proposed in the Statement of Work.

Rationale: Recent evidence indicates that subchronic administration of stimulants reduces basal extracellular dopamine (DA) concentrations and blocks acute stress-induced DA release in the nucleus accumbens (NA) of rats during withdrawal. However, no previous studies have attempted to relate chronic drug

exposure to stress reactivity and changes in DA transmission during early withdrawal.

Results: The data are illustrated in described in **Appendix 7**. **Appendix 7** is a galley proof of a paper in press in the *Psychopharmacology*. Figure numbers in the text below refer to the Figure numbers in the manuscript. Statistical analyses are reported in the preprint.

Subchronic METH increased DAT (Fig. 2) but not NET immunoreactivity in the NA compared to the STR and mPFC. METH reduced basal extracellular DA and blocked restraint-stress induced DA release in the NA SHELL (Fig. 3). DA uptake blockade increased extracellular DA more in the NA SHELL of METH rats (Fig. 4), whereas NE uptake blockade increased basal DA concentrations to a similar extent in METH and SAL rats (Fig. 5). Postmortem evaluation of dopamine tissue content in NA, STR or mPFC revealed no changes from saline controls.

Discussion:
The finding that subchronic administration of METH produced a significant increase in NA DAT immunoreactivity (Figure 2) when measured 1 day after the drug administration regimen is consistent with the hypothesis that chronic administration of METH reduces basal extracellular DA concentrations in the NA during early withdrawal.

DAT immunoreactivity after an early withdrawal period is significantly increased in the NA while basal DA concentrations in the NA SHELL (Figure 3,5) are reduced. Thus, it is possible that the increases in DAT protein may account for the enhanced uptake and lower basal concentrations of extracellular DA. The enhanced uptake of DA by NET in the NA SHELL may also contribute to the observed decreases in extracellular DA in METH-pretreated rats due to the heterotransport of DA by NET in this subregion (Yamamoto and Novotney 1998). However, this possibility is unlikely due to the finding that subchronic METH exposure did not increased NET immunoreactivity in the NA (see results). It should be noted that we did not distinguish DAT and NET immunoreactivities in the NA SHELL and NA CORE subregions of the NA. Moreover, since NET is localized almost exclusively to the NA SHELL (Berridge et al. 1997; Delfs et al. 1998) and the changes in basal extracellular DA were restricted to this subregion and not the NA CORE, the decreases in basal extracellular DA after subchronic METH are likely the result of increases in DAT and not NET protein in the NA SHELL.

These results also illustrate that not all dopaminergic terminal brain regions are affected equally by subchronic METH exposure. METH exposure selectively increased DAT in the NA compared to the STR and mPFC (Figure 2) and did not significantly change NET immunoreactivity in mPFC, hippocampus or NA of rats during early withdrawal.

The effects of local GBR12909 perfusion in the NA CORE and NA SHELL were also different and suggest that not only is there a pre-existent differential effect on extracellular DA in response to DAT blockade in NA SHELL compared to NA CORE, but that subchronic METH exposure selectively altered the DAT dependent regulation of extracellular DA in the NA SHELL (Figure 4).

Results from Experiment 2 (Figure 4) suggest that there is differential regulation of basal extracellular DA concentrations in the NA SHELL after a 24 hr withdrawal from subchronic METH exposure. The finding that blockade of DAT after subchronic low dose METH exposure results in *enhanced* increases in extracellular DA concentrations in the NA SHELL is consistent with the augmented increases in basal extracellular DA concentrations observed during local administration of GBR12909 in chronic cocaine exposed rats (Pierce and Kalivas 1997).

The finding that the local administration of the NET inhibitor nisoxetine increased basal extracellular concentrations of DA in the NA SHELL (Figure 5) is consistent with previous work showing that local administration of the NET inhibitor, desipramine increased extracellular DA concentrations in the NA SHELL of rats *in vivo* (Yamamoto and Novotney 1998). Although some studies have shown that NET binding increased in the bed nucleus stria terminalis of chronic cocaine-exposed non-human primates (Macey et al. 2003) and that norepinephrine is involved in stress-induced drug relapse (Shaham et al. 2000; Erb et al. 2000; Stewart 2000; Leri et al. 2002), no studies have examined the effects of subchronic METH exposure on NET protein immunoreactivity in the NA. The present study shows that the decreases in basal extracellular DA in the NA SHELL produced by subchronic METH are not due to the enhanced activity of NET (Figure 5) or an increase in NET protein.

In summary, these data illustrate that subchronic METH exposure selectively increases DAT but not NET immunoreactivity in the NA of rats. Moreover, subchronic METH exposure selectively reduces DA function in the NA SHELL compared to the NA CORE as evidenced by reduced basal DA concentrations, a blockade of stress-induced DA release, and an augmented but short-lived responsiveness to DAT uptake blockade in the NA SHELL. These findings have significance for understanding how repeated METH administrations selectively change mesoaccumbens DA transmission during early withdrawal and the ability to cope with stress. Given that reduced mesolimbic DA function is associated with negative affective disorders such as drug craving (Koob and Le Moal 1997), these data may also explain why a considerable population of METH abusers relapse during early withdrawal (Brecht et al. 2000; Brecht et al. 2004).

KEY RESEARCH ACCOMPLISHMENTS

- Chronic unpredictable stress enhances the neurotoxicity of methamphetamine to dopamine terminals
- Chronic restraint stress elevates basal extracellular glutamate concentrations in the hippocampus and may mediate the damage to this area.
- Chronic stress enhances the hyperthermic responses to the $5HT_{2A/C}$ agonist, DOI and thus may indicate that chronic stress can exacerbate hyperthermia via a sensitization of 5HT2 receptors.
- Methamphetamine increases GABA release in the substantia nigra via the D1 receptor and indicates the direct output pathway of the basal ganglia is activated. The increase in GABA release in the substantia nigra plays a critical role in the long-term loss of dopaminergic innervation to the striatum following methamphetamine.
- Methamphetamine produces protein oxidation and excitotoxicity in the striatum as evidenced by nitrotyrosine immunoreactivity and spectrin proteolysis, respectively. The spectrin proteolysis is blocked by an AMPA antagonist. Prior exposure to stress augments the METH-induced nitrotyrosine formation.
- Methamphetamine produces a bioenergetic compromise as evidenced by a decrease in complex II activity and protein.
- An experimental design and methodology was established to study *the in vivo* regulation of the glutamatergic projection from the subthalamic nucleus to innervate the substantia nigra. METH in combination with the antagonism of D2 receptors in the SN elevates the extracellular concentrations of glutamate and produces a depletion of dendritic spectrin and cell loss in the SN.
- Low dose subchronic METH exposure selectively increases nucleus accumbens dopamine transporter and reduces both basal and stress-induced DA release in the nucleus accumbens SHELL during early withdrawal without producing long-term changes in dopamine content. Thus, although low doses of METH do not produce evidence of toxicity, they do alter stress reactivity as evidenced by changes in dopamine transmission within the nucleus accumbens.

REPORTABLE OUTCOMES

Published papers during funding period

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Submitted Papers

Hatzipetros, T and Yamamoto, B.K. Dopaminergic Regulation of the Rat Subthalamonigral Glutamate Pathway, submitted to *Brain Research*

Abstracts/Meeting Presentations during funding period

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Hadjipetros, TA , Mintz, IM, and Yamamoto, BK Subthalamic stimulation-induced changes in extracellular glutamate in the rat substantia nigra: An in vivo microdialysis study. Society for Neuroscience, 2002

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CONCLUSIONS

The experiments conducted in accordance with the Statement of Work revealed several interesting outcomes. The results illustrated in this progress report support the conclusion that chronic environmental stress can potentiate the neurotoxic effects of methamphetamine on dopamine terminals. The mechanisms underlying the enhanced vulnerability produced by stress on methamphetamine-induced damage to dopamine neurons may include excitotoxicity mediated by an enhanced release of glutamate in the striatum or substantia nigra. The additive or synergistic effects of stress and methamphetamine-induced insults may enhance the vulnerability to the development of Parkinson's disease.

In addition, other findings described in this progress report have begun to characterize how the release of dopamine within the substantia nigra can alter the outflow of the basal ganglia through the activation of D1 receptors that in turn, could lead to an increase in corticostriatal glutamate release via a polysynaptic process to culminate in excitotoxic damage (spectrin proteolysis) to dopamine terminals in the striatum and through D2 receptor inhibition within the substantia nigra to disinhibit glutamate release and damage dopamine soma.

We have established a methodology to stimulate glutamate efferents from the subthalamic nucleus that innervate the substantia nigra while simultaneously measuring glutamate release. As a result, we are now positioned to determine if

(1) chronic stress exacerbates this increase in glutamate release and enhances the vulnerability of dopamine soma in the substantia nigra to excitotoxicity and oxidative damage and (2) the regulatory mechanisms of the glutamatergic efferents of the subthalamic nucleus to the substantia nigra. Overall, these studies continue to address the hypothesis that the synergistic interaction between chronic stress and methamphetamine damages dopamine neurons at both the terminal (striatum) and cell body (substantia nigra).

Address of Final Report Review:

Several issues were raised in the Final Report Review.

1. There were multiple mentions that several of the studies were reported in previous reports. That is indeed the case but my reasoning was that the previous reports were "works in progress" that were continued into and finalized in subsequent years.
2. CONTRACTUAL ISSUES were raised due to presumed budgetary overlap with my NIH grants. None of my NIH grants involved stress of any sort with the exception of the postdoctoral fellowship award to Dr. Matsuzewich (DA0597). However, this fellowship grant only provides salary support and no supply costs. Other grants were mentioned because some of the authors' salaries, and primarily mine, were derived from multiple sources. Thus, it was imperative that I acknowledge all funding sources from which our salaries were derived. I did not intend to prioritize the funding sources on the basis of percent funding contribution when I listed them in the acknowledgement section of the publications. The order of listing was random although perhaps it should have been prioritized.
    a. One of the grants (DA7427) is related to MDMA and is not related to the DoD project although some of the supplies purchased from DA7427 were used to support the oxidative stress assays.
    b. The other grant (DA07606) is related to methamphetamine and corticostriatal glutamate. There is no conceptual scientific overlap with the DoD project other than the elucidation of the excitotoxic effects of methamphetamine. Thus, supply costs with regard to the glutamate assays in both assays were shared and thus, both grants were appropriately acknowledged.
    c. The DA0597 grant was a postdoctoral fellowship grant on chronic stress to Dr. Leslie Matuszewich that covered part of her salary while most of her effort was related to the DoD project . She spent considerable effort toward exposing the rats to chronic stress for projects in both grants.
    d. Another issue that must be considered is that the expenses for animal purchases and housing nearly doubled when I transferred to Boston University from Case Western Reserve University. As the proposed projects in the DoD award involved chronic treatments and protracted housing of the rats, the cost for the proposed experiments markedly increased despite a fixed budget. Thus, some of the supplies purchased from other budget sources were used to complete the DoD project. Therefore, those sources were

appropriately acknowledged. In addition salaries were significantly increased from the original budget due to the cost of living increase in Boston. Therefore, salaries were supplemented from other sources related to methamphetamine toxicity research but not directly related chronic stress.

3. The reviewer accurately summarizes the progress and accomplishments of the project on the bottom of page 2 of the Report Review. All of Objectives 1-4 have been completed with the exception noted that the effects and interaction between environmental stress and METH on oxidative stress have not been reported. Since the submission of the original Final Report, we have completed those studies showing the environmental stress enhances the ability of METH to produce nitration of tyrosine residues in the striatum (Fig. 11, page 24). Therefore, all objectives have now been completed.
4. There were TECHNICAL ISSUES noted related to the lack of discussion of the results and the statistical analyses of the data.
    a. In the revised Final Report, the results are now more thoroughly discussed.
    b. Consistent with the allowance that published papers and presentations can be substituted for detailed descriptions, references to the appropriate appendices of published or submitted papers are now made within the body of the report. Although the majority of body of the report contains text taken directly from the publications, it is formatted specifically for the final report.
    c. The use of carbachol in Objective 4 is now clarified.
    d. There is now discussion as to why spectrin breakdown products return toward control values 7 days after METH administration (page 28).
    e. The explanation for the reporting of the results related to the previous funding period is in #1 above. The results reported in this project were funded by this project and reported accordingly. The citation and acknowledgement of other funding sources is explained in #2 above.
5. There were FORMAT issues noted. The report contains an objective, a results section, and a discussion section. The body now contains reference numbers to the numbered appendices. Figures not illustrated in the appendices of published or submitted papers now contain legends as needed.
6. There was an issue related to Key Accomplishments that only the last accomplishment was obtained during the present reporting period. The Final report describes collected experiments that are now published that were not published at the time of the previous report (2003-2004). *The new results are as follows*:

    Figure 1 is new.

    Results illustrated in Figures 1, 4, 5, and 7 of **Appendix 3** are new since the last annual report.

    Figures 2, 3, and 4 of **Appendix 4** are new

    Figures 6, 7, and 11 of Objective 5 in the Body are new

Figures 1-7 in **Appendix 6** are new.

In addition, Figures 1-5 of **Appendix 7** are new since the last annual report.

REFERENCES

A majority of the references cited in the report are detailed in the respective appendices and publications as noted. Other references are noted below:

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Nash J.F. and Yamamoto, B.K. Methamphetamine neurotoxicity and striatal glutamate release: Comparison to 3,4-methylenedioxymethamphetamine. Brain Research 581: 237-243, 1992.

LIST OF APPENDICES

1. Matuszewich, L. and Yamamoto, B.K. Chronic Stress Augments the Acute and Long-term effects of methamphetamine. Neuroscience 124: 637-46, 2004.

2. Matuszewich, L, and Yamamoto, B.K Long-lasting effects of chronic stress on DOI-induced hyperthermia. Psychopharmacology 169: 169-175, 2003

3. Mark, K.A., Soghomonian, J-J, Yamamoto, B.K. High-Dose Methamphetamine Acutely Activates the Striatonigral Pathway to Increase Striatal Glutamate and Mediate Long-term Dopamine Toxicity. Journal of Neuroscience 24(50): 11449-11456, 2004.

4. Hatzipetros, T and Yamamoto, B.K. Dopaminergic Regulation of the Rat Subthalamonigral Glutamate Pathway

5. Brown, J.M., Quinton, M.S. and Yamamoto, B.K. Methamphetamine-Induced Inhibition of Mitochondrial Complex II: Roles of Glutamate and Peroxynitrite. J. Neurochemistry, in press.

6. Staszewski, R.D. and Yamamoto, B.K. Methamphetamine-Induced Spectrin Proteolysis in the Rat Striatum. Submitted

7. Broom, S.L. and Yamamoto, B.K. Effects of Subchronic Methamphetamine Exposure on Basal DA and Stress-induced Dopamine Release in the Nucleus Accumbens Shell of Rats. Psychopharmacology, in press.

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APPENDIX 1

Neuroscience 124 (2004) 637–646

CHRONIC STRESS AUGMENTS THE LONG-TERM AND ACUTE EFFECTS OF METHAMPHETAMINE

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Abstract—There is growing evidence that exposure to stress alters the acute effects of abused drugs on the CNS. However, it is not known whether stress augments the longer-term neurotoxic effects of psychostimulant drugs, such as methamphetamine. Methamphetamine at high doses decreases forebrain dopamine concentrations. The current study tested the hypothesis that 10 days of unpredictable stress augmented striatal dopamine depletions 7 days following four injections of either 7.5 or 10 mg/kg methamphetamine (1 injection every 2 h). Furthermore, to assess the effects of chronic stress on immediate responses to methamphetamine, extracellular striatal dopamine and methamphetamine concentrations, and rectal temperature were monitored during the methamphetamine injection regimen. Seven days following either a 7.5 mg/kg or 10 mg/kg methamphetamine injection regimen, male rats exposed to unpredictable stress showed greater depletions in striatal dopamine tissue content compared with non-stressed controls injected with methamphetamine. Stressed rats had increased hyperthermic responses and dopamine efflux in the striatum during the methamphetamine injections when compared with non-stressed control rats. Moreover, stressed rats had an increased mortality rate (33%) compared with non-stressed controls (16.7%) following four injections of 10 mg/kg methamphetamine. The enhanced acute and longer-term effects of methamphetamine in stressed rats was not due to a greater concentrations of methamphetamine in the striatum, as extracellular levels of methamphetamine during the injection regimen did not differ between the two groups.

In summary, exposure to 10 days of chronic unpredictable stress augments longer-term depletions of dopamine in the striatum, as well as acute methamphetamine-induced hyperthermia and extracellular dopamine levels. These findings suggest that chronic stress increases the responsiveness of the brain to the acute pharmacological effects of methamphetamine and enhances the vulnerability of the brain to the neurotoxic effects of psychostimulants.

Key words: dopamine, hyperthermia, neurotoxicity, psychostimulants, striatum, unpredictable stress.

There is increasing evidence that repeated exposure to environmental stressors can alter behavioral and neurochemical responses to drugs of abuse. Prior exposure to stress enhances locomotor activity following a systemic injection of amphetamine, morphine or cocaine (Campbell and Fibiger, 1971; Antelman et al., 1980; Herman et al., 1984; Robinson and Becker, 1986; Kalivas and Stewart, 1991; Deroche et al., 1993; Robinson and Berridge, 1993; Stewart and Badiani, 1993; Deroche et al., 1995). Likewise, drug-induced dopamine release in several forebrain regions is augmented by pre-exposure to stress (Kalivas and Duffy, 1989; Sorg and Kalivas, 1991; Hamamura and Fibiger, 1993; Rouge-Pont et al., 1995). Exposure to stress also facilitates the propensity of rats to self-administer drugs of abuse (for review see Piazza and LeMoal, 1998; Covington and Miczek, 2001) and several studies have reported that prior exposure to methamphetamine (METH) may augment subsequent stress-induced responses in rats and human METH users (Tsuchiya et al., 1996; Yui et al., 1999, 2001; Wallace et al., 2001).

It is unknown if exposure to stress enhances the vulnerability of the dopamine system to the neurotoxic effects of drugs of abuse, in particular, METH. METH can act as a neurotoxin to monoamine neurons when administered at high doses or repeatedly in rodents and non-human primates (Ricaurte and McCann, 1992; Gibb et al., 1993) as evidenced by decreases in the number of tyrosine hydroxylase immunoreactive fibers (Hotchkiss and Gibb, 1980), in the density of dopamine terminals and uptake sites (Bittner et al., 1981; Pu et al., 1994), and in the amount of dopamine in striatal tissue (Ricaurte et al., 1980; Stephans and Yamamoto, 1994). The alterations in these biochemical markers have been reported to endure for months and are most pronounced in the striatum (Seiden et al., 1975; Ricaurte et al., 1980; Bittner et al., 1981; Villemagne et al., 1998). In abstinent human METH users, decreases in the dopamine transporter (DAT) have been reported also in the striata (Volkow et al., 2001). Human drug use is frequently associated with high levels of chronic stress (Koob and LeMoal, 2001); however, it is impossible to assess the relative contribution of stress to the effects of psychostimulant use in humans. The current study will assess whether chronic stress enhances the longer-term damaging effects of METH to the striatum in the rodent model.

Several factors mediate amphetamine-induced neurotoxicity in non-stressed rodents. Hyperthermia, which accompanies high doses of amphetamines, appears to be important for the long-term damage in the striatum. Reducing the core body temperature by pharmacological or environmental manipulations decreases markers of dopa-

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Abbreviations: ANOVA, analysis of variance; DAT, dopamine transporter; EDTA, ethylenediaminetetraacetic acid; HPLC-EC, high performance liquid chromatography with electrochemical detection; METH, methamphetamine; PE, polyethylene.

0306-4522/04$30.00+0.00
doi:10.1016/j.neuroscience.2003.12.007

mine terminal damage following psychostimulant injections (Ali et al., 1994; Miller and O'Callaghan 1994; Malberg et al., 1996). Another factor that may contribute to the neurotoxic effects of METH is the massive release of dopamine in the striatum during a neurotoxic injection regimen. Blocking METH-induced dopamine release with concurrent administration of dopamine uptake blockers attenuates dopamine depletions, as does inhibiting tyrosine hydroxylase synthesis (Schmidt et al., 1985; Marek et al., 1990; Pu et al., 1994). Chronic stress may influence either or both of these factors to potentiate METH-induced dopamine damage in the brain.

Therefore, the present study investigated the effects of chronic unpredictable stress on the longer-term neurochemical changes in the striatum associated with high doses of METH. We hypothesize that exposure to 10 days of stress will enhance the vulnerability of striatal neurons to METH neurotoxicity as evidenced by greater dopamine depletions. In addition, rectal temperature and acute dopamine release or METH concentrations in the striatum will be monitored during the METH injection regimens to assess any differences between these factors in stressed and non-stressed rats. Previous studies have reported that chronic unpredictable stress augments the motivational and locomotor responses to psychostimulant drugs (Prasad et al., 1998; Haile et al., 2001). The current experiments used the same stress procedure for 10 days, varying the type and timing of different moderate stressors to assess its effects on psychostimulant-induced neurotoxicity.

EXPERIMENTAL PROCEDURES

Animals and stress exposure

Male Sprague–Dawley rats (175–250 g) were purchased from Zivic Miller Laboratories (Allison Park, PA, USA). Rats were pair housed until intracranial surgery with food and water available *ad libitum*, on a 12-h light/dark cycle (lights on at 06:00 h and off at 18:00 h) in a temperature-controlled room (22 °C). All procedures were in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and approved by the local institutional animal care committee. For these studies, efforts were made to minimize the number of rats used and their discomfort throughout the experimental procedures.

Stressed rats were exposed to stressors that varied by day and time for 10 days (Stein-Behrens et al., 1994; Fitzgerald et al., 1996; Haile et al., 2001; Matuszewich and Yamamoto, 2003). The following schedule was followed for rats used in the microdialysis experiments: day 1 11:00 h 50 min cold room (4 °C), and 13:00 h 60 min cage rotation; day 2 13:00 h 4 min swim stress (23 °C), and 18:00 h lights on overnight (12 h); day 3 12:00 h 3 h lights off, and 15:00 h 60 min restraint stress; day 4 18:00 h 50 min cage rotation, and food and water deprivation overnight (14 h); day 5 15:00 h 15 min cold room isolation, and 16:00 h isolation housing overnight; day 6 11:00 h 3 min swim stress, and 15:00 h 60 min restraint stress; day 7 intracranial surgery; day 8 10:00 h 20 min cage rotation, and 15:00 h 2 h lights off; day 9 10:00 h 3 min swim stress, and 18:00 h food and water deprivation overnight; day 10 12:00 h 3 h lights off, and 18:00 h lights on overnight. Unstressed and stressed rats were weighed daily and both groups underwent intracranial surgery on day 7, as listed above.

For the hyperthermia, corticosterone, and ex vivo tissue experiments that did not require intracranial surgery, the same stress procedure was followed until day 6, after which the following schedule was used: day 6 11:00 h 3 min swim stress, and 15:00 h 2 h lights off; day 7 13:00 h 30 min cage rotation, and 18:00 h 1 h lights on; day 8 10:00 h 20 min cage rotation, and 15:00 h 60 min restraint stress; day 9 10:00 h 3 min swim stress, and 18:00 h food and water deprivation; day 10 18:00 h isolation housing and lights on overnight.

Experiment 1: METH treatment of stressed and non-stressed rats

Drug injections and temperature measurements. On day 11, i.p. injections of 7.5 mg/kg or 10 mg/kg *d*-METH hydrochloride salt (National Institute of Drug Abuse, Bethesda MD, USA), or an equivalent volume of saline (0.9% NaCl), were given every 2 h for a total of four injections. METH was dissolved in saline and given in a volume of 1 ml/kg. The high METH dosing regimen (10 mg/kg×four injections) was selected due to its reliability for causing long-term dopamine depletions in the striatum (Stephans et al., 1998). The lower dosing regimen (7.5 mg/kg×four injections) was selected due to its lower mortality rate. Rectal temperature was measured 30 and 60 min following each i.p. injection with a Thermalert TH-8 monitor (Physitemp Instruments, Inc., Clinton, NJ, USA) by holding each rat at the base of the tail and inserting a probe (RET-2) 4.6 cm past the rectum into the colon for 6–8 s until a rectal temperature was maintained for 3 s. If a rat had a rectal temperature greater than 41.0 °C, wet ice was placed in a tray underneath the cage for 30 min.

Microdialysis procedures. Rats used in the microdialysis experiments were anesthetized with a combination of xylazine (6 mg/kg) and ketamine (70 mg/kg) and placed into a Kopf stereotaxic frame. The skull was exposed and a 21-gauge stainless steel guide cannula (11 mm in length; Small Parts, Inc., Miami Lakes, FL, USA) was positioned above the striatum (+2.0 mm anterior and ±3.2 mm medial to bregma). The cannula and a metal female connector were secured to the skull with three stainless steel screws and cranioplastic cement. An obturator fashioned from 31-gauge stainless steel wire, ending flush with the guide cannula, was inserted into the cannula after surgery.

Four days following surgery, the obturator was removed from the guide cannula and replaced with a microdialysis probe. The microdialysis probes were constructed as previously described (Lowy et al., 1993) from a 27-gauge thin wall stainless steel tube, fitted with a dialysis membrane (13,000 dalton cutoff; 210 μm o.d.; Spectrum Laboratories, Inc., Rancho Domingues, CA, USA) at one end, and a 3 cm piece of polyethylene (PE) 20 tubing (Fisher Scientific, Inc., Pittsburg, PA, USA) at the other end, to serve as the inlet for the perfusion medium. The dialysis membrane was 4 mm×210 μm diameter. A 4 cm length of capillary tubing (125 μm o.d., 50 μm i.d.; Polymicro Technologies, Phoenix, AZ, USA) served as the outlet from the dialysis membrane. The vertical placement of the microdialysis probe was determined during construction of the probe by gluing a ring of PE 20 tubing, which acts as a mechanical "stop," at a measured distance along the length of the probe. The positioning permitted the exposed portion of the dialysis membrane to extend beyond the guide cannula and into the striatum (ventral from dura −1.0 to −5.0). The rats were placed in microdialysis cages and attached via a spring-covered tether to a swivel (Instech Laboratories, Inc., Plymouth Meeting, PA, USA). Dulbecco's phosphate-buffered saline medium (NaCl 138 mM, 2.1 mM KCl, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 8.1 mM NaH_2PO_4, 1.2 mM $CaCl_2$, and 5 mM *d*-glucose, pH 7.4) was pumped immediately through the microdialysis probe with a Harvard Model 22 syringe infusion pump (Hollison, MA, USA) at a rate of 2.0 μl/min. After a 3 h equilibration period, the following 1 h samples were collected: two baseline samples and eight samples during systemic METH injections.

High performance liquid chromatograph. Microdialysis samples from the striatum were assayed for dopamine by high performance liquid chromatography with electrochemical detection (HPLC-EC). For catecholamine detection, samples (22 μl) were loaded via a Rheodyne injector (Cotati, CA, USA) onto a 3 μ C18 column (100×2 mm; Phenomenex, Torrance, CA, USA). The mobile phase (pH 4.2) consisted of 32 mM citric acid, 54.3 mM sodium acetate, 0.074 mM EDTA, 0.215 mM octyl sodium sulfate and 3% methanol. Compounds were detected with an LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA), with a 6 mm glassy carbon working electrode maintained at a potential of +0.7 V relative to an Ag/AgCl reference electrode. Data were collected using a Hewlett Packard Integrator (Palo Alto, CA, USA).

To assess METH concentrations in dialysate, 40 μl of sample was loaded via a Rheodyne injector onto a 5 μ C18 column (150×2 mm; Phenomenex). A Hewlett Packard 1050 pump delivered 0.455 ml/min of mobile phase (50 mM NaH_2PO_4, 6% acetonitrile, pH=4.6) to the column. Striatal concentrations of METH were detected with a Waters 486 Tunable Absorbance Detector (Milford, MA, USA), with the wavelength set at 245 nm.

Analysis of dopamine tissue concentrations in striatal tissue was performed on an HPLC-EC, as described above. The tissues were sonicated in 0.1 M perchloric acid, centrifuged at 13,000×*g* for 6 min and the supernatant assayed for dopamine. The pellet was re-suspended in 1 N NaOH and protein content determined by a Bradford protein assay.

Histology

One week after systemic METH or saline injections, rats were killed and the brains were quickly removed from the skull and frozen on dry ice (Stephans and Yamamoto, 1994). Probe placements were verified from frozen coronal sections and only rats with probes located in the striatum were used for statistical analysis. Striatal tissue was dissected from a 400 μm slice. The tissue was frozen at −80 °C for later analysis of neurotransmitter content.

Experiment 2: Endocrine measures of stress exposure

On the 11th day, trunk blood and adrenal glands were collected from stressed and non-stressed rats that did not have microdialysis cannula implanted or injections of either saline or METH. All rats were rapidly decapitated between 10:00 and 11:00 h. Trunk blood was collected into a 15 ml vial with 0.3 ml heparin sodium sulfate (1000 U/ml), centrifuged for 15 min (4000×*g*) and the plasma frozen until assayed. From the same rats, adrenal glands were collected and weighed immediately. Corticosterone was measured in 5 μl plasma samples that were diluted with 100 μl of sterile water. The radioimmunoassay employed [^{125}I]corticosterone from ICN Pharmaceuticals (Costa Mesa, CA, USA) and antisera from Ventrex (Portland, ME, USA; Matuszewich et al., 2002). The intra- and inter-assay coefficients of variation were less than 10% with a detectable assay range of 0.1–400 μg corticosterone/100 ml plasma.

Statistical analysis

Independent *t*-tests were used to compare body weight differences (weight on day 11−weight on day 1), corticosterone concentrations, adrenal weights and baseline concentrations of dopamine from chronically stressed rats and non-stressed controls. Adrenal weights were standardized to 100 g of body weight. Two-way, repeated measures analyses of variance (ANOVA) were computed to compare dialysate dopamine or METH concentrations or rectal temperatures over time. Dopamine concentration in dialysate was compared in 10 samples (two predrug samples and eight samples during systemic administration of METH). METH concentration in dialysate was compared in nine samples (one predrug sample and eight samples during systemic administration of METH) because there was no detectable level of METH in the striatum prior to drug injection. Rectal temperature was compared at nine time points (30 min before the first drug injection and 30 and 60 min after each systemic administration of METH). Dopamine tissue concentrations between chronically stressed rats and non-stressed controls were compared in a two-way ANOVA. Post hoc Tukey's pairwise tests were used to analyze any significant treatments. Statistical significance was fixed at $P<0.05$ for all tests.

Table 1. Effect of chronic stress on the body weight, adrenal weight and corticosterone levels[a]

	Non-stressed rats	Chronically stressed rats
Body weight (g) (difference day 11-day 1)	71.42±3.67	39.51±3.26*
Adrenal weight (mg/100 g body weight)	11.35±.99	13.55±.73$^+$
Corticosterone (trunk blood μg/dl)	0.89±.60	0.79±.41

[a] All data are presented as the mean+S.E.M. * $P<.01$ when compared to non-stressed rats by an independent Student's *t*-test. $^+$ $P<.10$ when compared to non-stressed rats by an independent Student's *t*-test.

RESULTS

Effects of chronic stress on body weight, adrenal weight and basal corticosterone levels

Across all experiments, rats exposed to the 10 day stress protocol ($n=49$) showed lower total body weight gain compared with non-stressed controls ($n=47$; $t(94)=6.549$, $P<0.01$; Table 1). There was no statistical difference between stressed and non-stressed adrenal gland weights ($t(18)=1.78$, $P<0.10$) or basal plasma corticosterone levels ($t(18)=0.145$; Table 1). However, of the 10 stressed and 10 non-stressed rats, corticosterone levels were only detectable in four plasma samples of each group.

Effects of chronic stress on METH-induced mortality and hyperthermia

Of the rats exposed to chronic stress, eight of 24 rats that received four METH injections of 10 mg/kg died (33.3%) compared with four of 24 of the non-stressed controls (16.7%). Two of 10 chronically stressed rats died following 7.5 mg/kg METH (20%), while one of 10 non-stressed control rats died (8.3%). None of the rats injected with saline from either group died.

Both stressed and non-stressed rats showed overall increases in rectal temperature after METH injections (7.5 mg/kg: $F(8,128)=8.81$, $P<0.05$; 10 mg/kg: $F(8,56)=13.79$, $P<0.05$). However, chronically stressed rats showed higher rectal temperatures during either 7.5 or 10 mg/kg METH injection regimens compared with non-stressed controls (7.5 mg/kg: $F(8,128)=2.51$, $P<0.05$, Fig. 1a; 10 mg/kg: $F(8,56)=4.56$, $P<0.05$, Fig. 1b). The rectal temperature of stressed rats peaked at 39.2 °C compared with 38.5 °C for non-stressed rats during the four injections of 7.5 mg/kg METH. Likewise, during injections of 10 mg/kg METH, peak rectal temperature of stressed rats reached 40.6 °C while control non-stressed rats reached 39.6 °C.

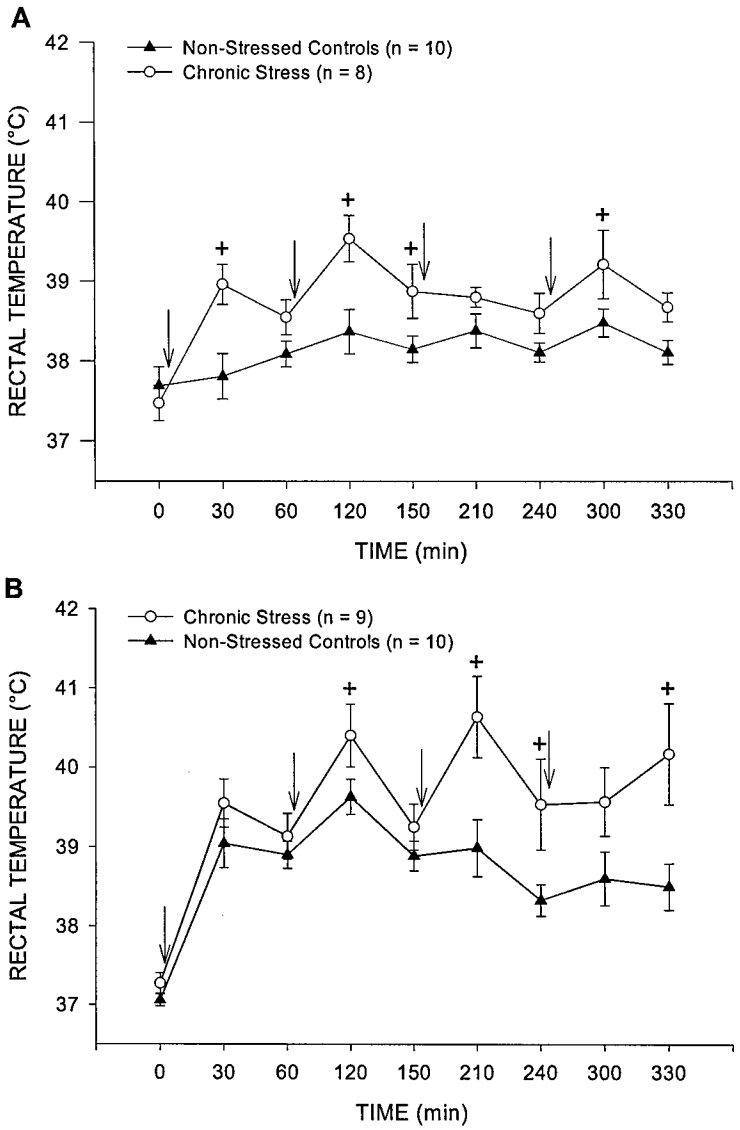

Fig. 1. Rectal temperature increased during injections of (a) 7.5 mg/kg or (b) 10 mg/kg METH (i.p., every 2 h for a total of four injections) compared with temperatures prior to the 1st injection (time=0). Rats exposed to chronic unpredictable stress showed greater hyperthermic responses compared with non-stressed controls ($^{+}$ $P<0.05$). Values are expressed as means±S.E.M. Arrow indicates each injection of METH.

Effects of chronic stress on dopamine in the striatum

Extracellular levels of dopamine increased after injections of 7.5 or 10 mg/kg METH (7.5 mg/kg: $F(9,126)=49.9$, $P<0.05$, Fig. 2a; 10 mg/kg: $F(9,90)=35.02$, $P<0.05$, Fig. 2b). Peak dopamine content in dialysis samples was dose-dependent with dopamine concentrations peaking at 66 pg/20 μl following 7.5 mg/kg×four METH, and 110 pg/20 μl following 10 mg/kg×four METH. Chronically stressed rats injected with 10 mg/kg METH showed greater dopamine increases compared with non-stressed controls ($F(1,9)=2.78$, $P<0.05$; Fig. 2b). There was no difference in basal concentrations of dopamine in striatum between stressed (2.08±0.16 pg/20 μl) and non-stressed rats (2.07+0.11 pg/20 μl; $t(56)=0.02$, n.s.).

Prior exposure to stress potentiated METH-induced dopamine decreases in striatal tissue collected 7 days after 7.5 or 10 mg/kg METH. Dopamine concentrations in the striatum did not differ between stressed or non-stressed rats following saline injections ($t(28)=0.23$; stressed: 145.5±14.9 ng/mg protein; non-stressed: 141.1±11.8 ng/mg protein). Therefore, dopamine content is presented graphically as a percentage of the dopamine content in saline-injected striata. Four injections of 7.5 mg/kg METH decreased dopamine content of striatal tissue in stressed rats compared with non-stressed rats injected with METH ($F(1,35)=6.09$, $P<0.05$, Fig. 3a). Both non-stressed and stressed rats had lower striatal dopamine levels following four injections of 10 mg/kg METH relative to saline-injected rats ($F(1,31)=44.54$, $P<0.05$).

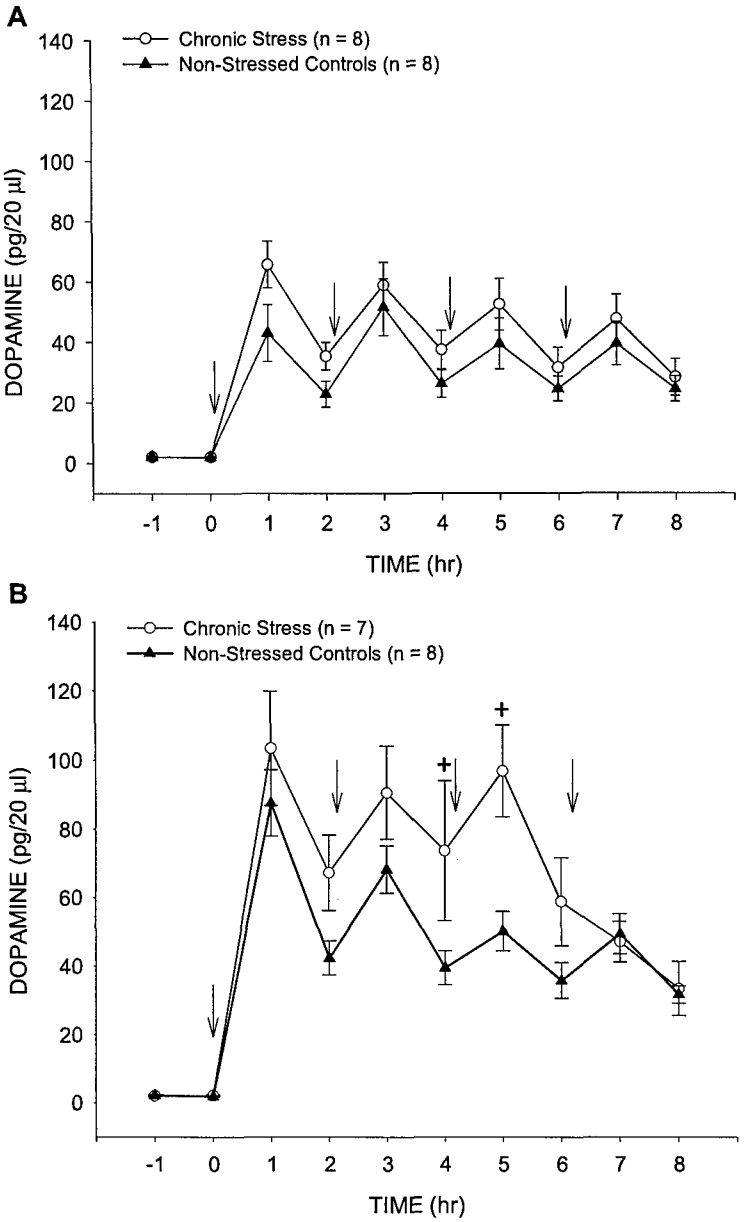

Fig. 2. *In vivo* extracellular dopamine concentrations in the striatum prior to and during injections of (a) 7.5 mg/kg or (b) 10 mg/kg METH (i.p., every 2 h for a total of four injections). METH increased dopamine levels for stressed and non-stressed rats relative to baseline concentrations. Rats exposed to chronic unpredictable stress showed greater increases of extracellular dopamine compared with non-stressed controls ($^{+}$ $P<0.05$). Values are expressed as means±S.E.M. Arrow indicates each injection of METH.

Dopamine concentrations in the striatum of stressed rats were significantly decreased compared with non-stressed rats treated with four injections of 10 mg/kg METH ($F(1,31)=4.195$, $P<0.05$, Fig. 3b).

Effects of chronic stress on METH concentrations in the striatum

The concentration of METH in the striatum increased following the four injections of 10 mg/kg METH in both stressed and non-stressed rats ($F(8,104)=15.65$, $P<0.01$; Fig. 4), peaking after the 3rd METH injection. However, there were no significant differences in the extracellular concentrations of METH during the injection regimen between stressed and non-stressed rats ($F(1,13)=0.11$, n.s.).

DISCUSSION

Chronic unpredictable stress augmented the neurotoxic effects of repeated METH injections. One week after administration of METH, decreases in striatal dopamine content were potentiated in rats exposed to chronic stress compared with non-stressed control rats. Systemic injec-

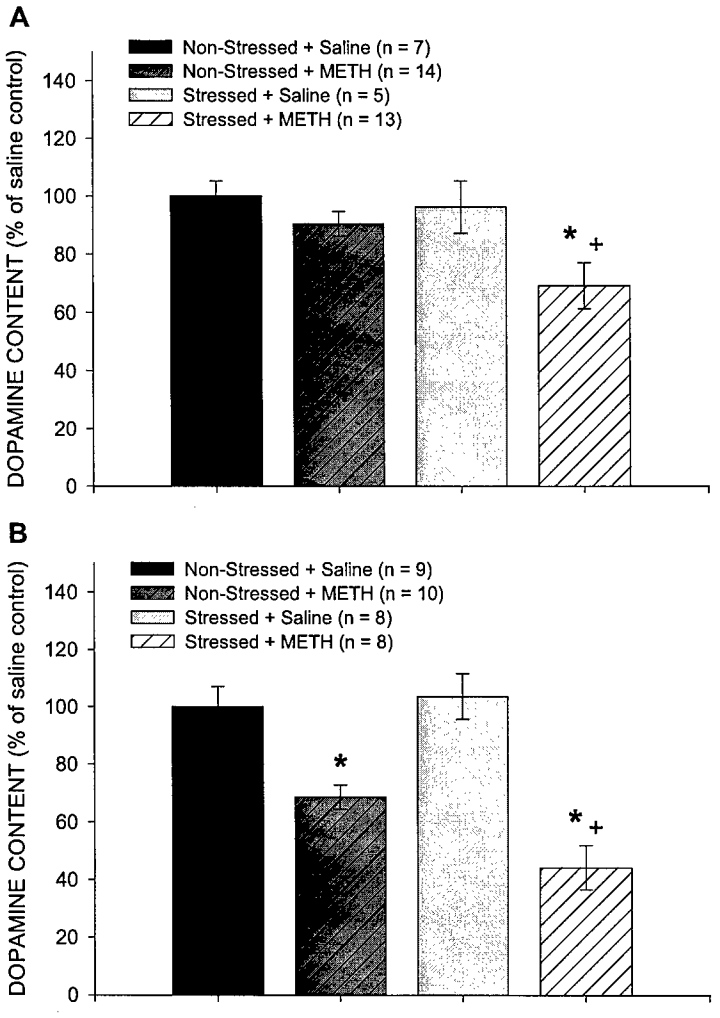

Fig. 3. Ex vivo dopamine concentrations in the striatum 7 days following injections of (a) 7.5 mg/kg or (b) 10 mg/kg METH (i.p., every 2 h for a total of four injections). Injections of 10 mg/kg METH depleted dopamine levels in the striatum for stressed and non-stressed rats relative to rats treated with saline (* $P<0.05$). Rats exposed to chronic unpredictable stress showed greater decrease of striatal dopamine content compared with non-stressed controls following 7.5 or 10 mg/kg METH ($^{+}$ $P<0.05$). Values are expressed as means±S.E.M.

tions of METH also elevated extracellular levels of dopamine in the striatum to a greater extent in chronically stressed rats than in non-stressed controls. Previous studies in our and other laboratories have shown acute increases in extracellular dopamine during METH administration and subsequent long-term depletions of striatal dopamine (Seiden et al., 1975; Ricaurte et al., 1980; Stephans and Yamamoto, 1994). However, to our knowledge, this is the first report that prior exposure to stress augments the depletion of striatal dopamine content 1 week after drug treatment, as well as the acute release of dopamine during high doses of METH.

Several factors proposed to mediate dopamine depletions following high doses of METH may be responsible for the observed potentiated depletions of dopamine in chronically stressed rats. A long-term depletion of dopamine content in the striatum after METH is correlated with elevated body temperatures during METH administration (Itoh et al., 1986; Bowyer et al., 1994). Pharmacological agents that lower body temperature attenuate METH-induced dopamine depletions in the striatum, as do lower ambient temperatures (Sonsalla et al., 1991; Bowyer et al., 1992, 1994). In the present study, stressed rats showed greater hyperthermia during METH administration (Fig. 1) and this increase in rectal temperature may contribute to the enhanced dopamine damage observed 1 week after METH injections (Fig. 3).

The increased hyperthermic response also may account for the elevated mortality of stressed rats observed during and following the 10 mg/kg METH injections. Thirty-three percent of the rats exposed to unpredictable stress died compared with 16.7% of the non-stressed rats injected with 10 mg/kg METH, even though cooling was used for both groups to decrease mortality. These results are consistent with the finding that hyperthermia mediates the lethal effects of amphetamines in rodents (Askew, 1962). Central 5-HT receptors contribute to temperature regulation in rats (Salmi and Ahlenius, 1998) and are in-

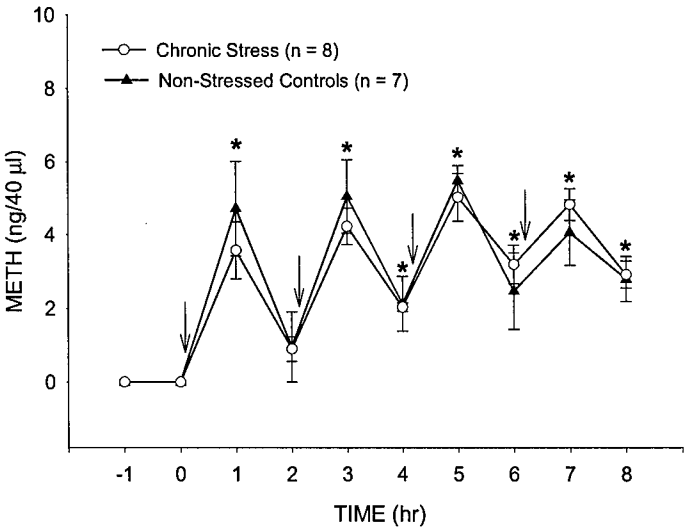

Fig. 4. *In vivo* extracellular METH concentrations in the striatum prior to and during injections of 10 mg/kg METH (i.p., every 2 h for a total of four injections). Striatal levels of METH increased for stressed and non-stressed rats relative to baseline concentrations (time=0; * $P<0.05$). There was no difference in METH concentrations between rats exposed to chronic unpredictable stress and non-stressed controls. Values are expressed as means±S.E.M. Arrow indicates each injection of METH.

creased following exposure to acute or chronic stressors (Papp et al., 1994; Ossowska et al., 2001). Following the 10-day unpredictable stress paradigm used in the current experiments, stressed rats showed a greater increase in rectal temperature after a systemic injection of a 5-HT_2 receptor agonist compared with non-stressed rats (Matuszewich and Yamamoto, 2003). Although stimulation of 5-HT_2 receptors may mediate the acute hyperthermia associated with METH, the relationship between 5-HT_2 receptor activation and longer-term neurotransmitter content changes after METH remains to be established. Pretreatment with the 5-HT_2 receptor antagonist ritanserin failed to prevent METH-induced decreases in tyrosine and tryptophan hydroxylase activities in the neostriatum, 1–20 h after the last METH injection (Johnson et al., 1988, 1994). Furthermore, although chronic treatment with desipramine has been shown to decrease 5-HT_2 receptor number or binding density in humans or rodents (Mason et al., 1993; Goodnough and Baker, 1994; Yatham et al., 1999), it did not reduce METH-induced dopamine depletions in either stressed or non-stressed rats (Matuszewich and Yamamoto, unpublished observations).

Alternatively, chronic stress may enhance METH-induced dopamine depletions in the striatum by increasing the acute release of dopamine. Extracellular dopamine concentrations were greater following METH injections in rats exposed to unpredictable stress, than in non-stressed controls (Fig. 2). Blocking dopamine transmission through inhibition of synthesis, blockade of transporter-mediated uptake or co-administration of dopamine antagonists attenuates METH-induced dopamine depletions (Buening and Gibb, 1974; Schmidt et al., 1985; Sonsalla et al., 1986; Marek et al., 1990; Pu et al., 1994). The acute increase in extracellular dopamine may contribute to longer-term dopamine depletions through the generation of free radical species and quinones (Cubells et al., 1994; Hirata et al., 1995; Huang et al., 1997; Yamamoto and Zhu, 1998; Fumagalli et al., 1999; Larsen et al., 2002).

The potentiation of METH-induced extracellular dopamine levels in rats exposed to stress parallels other findings following a challenge injection of amphetamine or cocaine (for review see Kalivas and Stewart, 1991). Repeated exposure to stress may contribute to the enhanced dopamine release by increasing tyrosine hydroxylase and/or the releasable stores of dopamine, inhibiting dopamine catabolism, decreasing dopamine uptake, or increasing impulse generation in dopaminergic neurons. Ortiz and colleagues (1996) reported an increase of tyrosine hydroxylase in the ventral tegmental area, but not the substantia nigra, following the same 10-day stress procedure as used for the current study. Although increases in dopamine synthesis may explain elevated extracellular dopamine concentrations in the mesolimbic terminal regions, such as the nucleus accumbens, other mechanisms may be operative in the nigrostriatal system (Beitner-Johnson et al., 1991, 1992; Beitner-Johnson and Nestler, 1991; Sorg and Kalivas, 1991).

Due to the ability of METH to release dopamine through reverse transport (Fischer and Cho, 1979), stress-induced alterations in the DAT may account for the augmented release of dopamine during METH injections. However, acute social stress in mice housed in isolation reduced DAT binding in the striatum (Isovich et al., 2001), as did exposure of male tree shrews to chronic subordinate stress (Isovich et al., 2001). These studies suggest that the observed increase in striatal extracellular dopamine in chronically stressed rats is not due to increases in DAT. Interestingly, elevated body temperature also can influence the function of DAT by increasing the intracellular accumulation of METH (Metzger et al., 2000; Xie et al.,

2000). Therefore, the augmented hyperthermic responses in the stressed rats (Fig. 1) may enhance the function of DAT and subsequently contribute to observed increases in extracellular dopamine during METH treatment, irrespective of the number of transporters.

The acute increases or delayed depletions of dopamine observed in the rats exposed to chronic stress do not appear to be due to increased bioavailability of METH in the striatum. METH concentrations in the striatum of stressed and non-stressed rats were similar throughout the METH injection regimen (Fig. 4). While METH concentrations measured with *in vivo* microdialysis suggest that the extracellular concentrations are similar between stressed and non-stressed rats, this technique does not assess the concentrations of METH in dopamine terminals. The concentration of METH in the terminals and subsequent alterations of vesicular pH gradients may be more critical to the longer-term dopamine depletions than extracellular METH concentrations (Sulzer and Rayport, 1990).

Overall, several mechanisms may contribute to the acute increases in hyperthermia or extracellular dopamine in the striatum and the potentiated decreases in dopamine tissue content. The precise effects of repeated, unpredictable stress on the brain are unknown but alterations in 5-HT receptors (Ossowska et al., 2001) or the dopaminergic system (Ortiz et al., 1996; Ossowska et al., 2001), may account for the enhanced hyperthermia, mortality, extracellular dopamine concentrations, or depletions of dopamine content in the striatum. Due to the broad overlap of stress and drug use (Piazza and LeMoal, 1998; Yui et al., 1999, 2001; Koob and LeMoal, 2001), the increased vulnerability of the brain by exposure to unpredictable stressors may be important for understanding the potential detrimental effects of drugs of abuse.

Acknowledgements—We thank Dr. D. A. Finn at the Veterans Affairs Medical Center and Department of Behavioral Neuroscience of Oregon Health and Science University for analyzing the plasma samples. This work was supported by National Institutes of Health grants DA07606 and DA05937-02 and by Department of Defense grant 17-99-1-9479.

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(Accepted 7 December 2003)

Psychopharmacology (2003) 169:169–175
DOI 10.1007/s00213-003-1498-7

ORIGINAL INVESTIGATION

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Long-lasting effects of chronic stress on DOI-induced hyperthermia in male rats

Received: 30 September 2002 / Accepted: 28 March 2003 / Published online: 27 May 2003

Abstract *Rationale:* Exposure to chronic stress can affect the serotoninergic (5-HT) system and behavioral measures associated with 5-HT. Repeated stress increases 5-HT receptor subtype 2 (5-HT_2) mediated behaviors in rodents, such as wet dog shakes and head twitch. *Objectives:* The current study investigated whether exposure to chronic unpredictable stress would augment 5-$HT_{2A/C}$ receptor-mediated hyperthermia. Furthermore, the persistence of these hyperthermic effects was investigated by testing rats up to 60 days after the stress procedure terminated. *Methods:* For 2 or 10 days, rats were either not stressed (controls) or exposed to chronic unpredictable stress, i.e. two stressors per day of the following: cage rotation, cold exposure, swim, restraint, light cycle manipulations, single housing, and food and water deprivation. After the termination of stress (day 3 or 11), the 5-$HT_{2A/C}$ receptor agonist DOI (1.5 mg/kg) or saline, was injected and the rectal temperature of the rats was monitored. In a separate experiment, the 5-HT_2 receptor antagonist, LY-53,587, was injected 30 min prior to the injection of DOI or saline. Finally, DOI was injected into rats 8, 30 or 60 days after the 10-day stress procedure ended. *Results:* Rats exposed to 10 days, but not 2 days, of unpredictable stress exhibited higher rectal temperatures following DOI than non-stressed rats. The DOI-induced hyperthermia was attenuated by LY-53,587. The augmentation of DOI-induced hyperthermia in stressed rats persisted when examined 8, 30 and 60 days following the stress procedure. *Conclusions:* The enhancement of 5-HT receptor function by chronic stress persists even after the environmental stressor is removed. This lasting increase in 5-HT receptor function may have implications for clinical disorders associated with stress, such as depression or post-traumatic stress disorder.

Keywords Chronic stress · Serotonin · 5-HT_2 receptor · Hyperthermia

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Introduction

Acute or chronic stress is known to produce alterations in the serotonin (5-HT) neurotransmitter system. Specifically, repeated exposure to social or physical stressors increases the density of the 5-HT subtype 2A (5-HT_{2A}) receptor in the cortex (Torda et al. 1988; Davis et al. 1995; McKittrick et al. 1995; Takao et al. 1995; Berton et al. 1998; Ossowska et al. 2001). Increases in the density or affinity of the 5-HT_{2A} receptor also have been shown to consequently augment 5-HT-mediated behaviors. Stimulation of the 5-HT_{2A} receptor by the 5-$HT_{2A/C}$ receptor agonist, (±)1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), altered temperature regulation, wet dog shakes and head shakes in rats or mice (Yap and Taylor 1983; Goodwin et al. 1984; Watson and Gorzalka 1990, 1992; Granoff and Ashby 1998; Lin et al. 1998; Salmi and Ahlenius 1998), and exposure to psychosocial stress or repeated electric foot shock further augmented DOI-stimulated wet dog or head shakes (Metz and Heal 1986; Gorzalka et al. 1998).

Chronic, unpredictable stress exposes a rat to different stressors, rather than the same stressor, over a number of days (10–31). Previous papers have suggested that chronic unpredictable stress produces a valid model of human depression due to its similarity in etiology, symptoms and treatment response (Katz et al. 1981;

Willner et al. 1992; Papp et al. 1996). Chronic, unpredictable stress has been shown also to increase the number of cortical 5-HT_{2A} receptors (Papp et al. 1994; Ossowska et al. 2001), but the behavioral relevance of these changes is unknown. Furthermore, few studies have examined the behavioral or pharmacological responsiveness of the 5-HT system following the termination of stress, which may provide insight into the longer-term changes associated with stress-induced illness such as depression.

Since DOI has been shown to increase body temperature in rats (Pranzatelli and Pluchino 1990; Lin et al. 1998; Salmi and Ahlenius 1998), the present study investigated whether chronic unpredictable stress augments DOI-induced hyperthermia. To assess the persistence of behavioral changes associated with the 5-$HT_{2A/C}$ receptor, hyperthermia was assessed for up to 2 months following the termination of the chronic, unpredictable stress procedure in stressed and control rats.

Materials and methods

Animals and stress exposure

Male Sprague-Dawley rats (175–250 g) were purchased from Zivic Miller Laboratories (Allison Park, Pa., USA). Rats were pair housed in 36×26.5×21.5 cm plastic cages with Santi-Chips covering the cage floor in a temperature-controlled room (mean room temperature=22°C). Rat chow (Purina Mills, Inc., Richmond, Ind., USA) and water (tap) were available without restrictions unless specified by the stress protocol. The rats were maintained on a 12/12 h light/dark cycle with lights on at 6 a.m. and off at 6 p.m. All procedures were in adherence to the National Research Council's Guide for the Care and Use of Laboratory Animals (1996) and approved by the local institutional animal care committee.

Rats assigned to the stress condition were exposed to stressors that varied by day and time for either 2 or 10 days (Stein-Behrens et al. 1994; Fitzgerald et al. 1996; Haile et al. 2001). The following procedure was used for rats exposed to 2 days of stress: day 1 10:00 a.m. 3-min swim stress (23°C), and 6:00 p.m. food and water deprivation (14 h); and day 2 6:00 p.m. single housing and lights on overnight (12 h). The following procedure was used for rats exposed to 10 days of stress: day 1 11:00 a.m. 50-min cold room (4°C), and 12:00 p.m. 60-min cage rotation; day 2 1:00 p.m. 4-min swim stress (23°C), and 6:00 p.m. lights on overnight (12 h); day 3 12:00 p.m. 3-h lights off, and 3:00 p.m. 60-min restraint stress (6×21.6 cm; Harvard Apparatus, Inc. Holliston, Mass., USA); day 4 6: 00 p.m. 50-min cage rotation, and food and water deprivation overnight (14-h); day 5 3:00 p.m. 15-min cold room, and 4:00 p.m. single housing overnight; day 6 11:00 a.m. 3-min swim stress, and 3:00 p.m. 2-h lights off; day 7 1:00 p.m. 30-min cage rotation, and 6:00 p.m. 1-h lights on; day 8 10:00 a.m. 20-min cage rotation, and 3:00 p.m. 60-min restraint stress; day 9 10:00 a.m. 3-min swim stress, and 6:00 p.m. food and water deprivation; day 10 6:00 p.m. single housing and lights on overnight. Stressed and non-stressed rats were weighed daily to monitor their overall health.

Drug injections and temperature measurements

On the test day, all rats were moved to an observation room for a period of 4 h to allow for the stabilization of body temperatures. Ambient temperature of the observation room was 26–27°C. Saline (1 ml/kg) or 1.5 mg/kg DOI hydrochloride (Sigma RBI, St Louis, Mo., USA) intraperitoneal (IP) injections were given to non-stressed and stressed rats. Rectal temperatures were measured 30 min before and 30, 45, 60, 75, 90 and 120 min after the systemic injection with a Thermalert TH-8 monitor (Physitemp Instruments, Inc., Clinton, N.J., USA). For the rectal temperatures, each rat was held by the base of the tail and a probe (RET-2) was inserted 4.6 cm past the rectum into the colon for 6–8 s until a rectal temperature was maintained for 3 s. For rats that were tested following 2 days of chronic unpredictable stress, rectal temperatures were taken at 30 min before and 30, 60, 90 and 120 min after the injection of DOI. In a separate experiment, the 5-HT_2 receptor antagonist LY-53,857 maleate (2.1 mg/kg; Sigma RBI, St Louis, Mo., USA) or vehicle was injected IP, 30 min prior to the injection of saline or DOI (1.5 mg/kg), immediately following the first rectal temperature. The number of rats used in each group was as follows: pretreated with vehicle and injected with saline, n=14 control (CVS), n=14 stressed (SVS); pretreated with vehicle and injected with DOI, n=21 control (CVD), n=20 stressed (SVD); pretreated with LY-53,857 and injected with saline, n=8 control (CLS), n=8 stressed (SLS); and pretreated with LY-53,857 and injected with DOI, n=7 control (CLD), n=7 stressed (SLD).

Stressed and control rats were injected with 1.5 mg/kg DOI at 8, 30 or 60 days following the chronic stress procedure to assess 5-$HT_{2A/C}$ receptor mediated hyperthermia over time. On these test days, the same procedures were used as described above and all rats received 1.5 mg/kg DOI. Rectal temperature was measured 30 min before and 30, 60, 75, 90 and 120 min after the DOI injection. The number of rats used in each group was as follows: day 8, n=13 stressed, n=14 control; day 30, n=8 stressed, n=9 control; and day 60, n=11 stressed, n=11 control.

Statistics

Body weights of stressed and non-stressed controls were compared using an independent t-test on days 1 and 11. Mixed factorial analysis of variances (ANOVAs) were used to compare pretreatment (stress versus control) and drug injection (Vehicle+DOI, Vehicle+Vehicle, or LY-53,857+Vehicle, versus LY-53,857+DOI) on rectal temperatures across time; and pretreatment and day tested (8, 30 or 60) on rectal temperatures across time. Significant effects and interactions of pretreatment (stress versus control) were further investigated with two-way ANOVAs followed by Tukey post hoc comparisons. All data are summarized by computing the area under the curve (AUC; summation of the rectal temperature taken at each test time point subtracted from the pre-injection temperature). AUC data for Figs 1, 2 and 3 were analyzed with a one-way ANOVA and data for Fig. 4 were analyzed with a two-way ANOVA (group versus day of test).

Results

Effects of DOI immediately following chronic stress

Stressed and non-stressed control rats did not differ in body weight at the start of the stress protocol [stressed: 213.1±2.6 g; controls: 215.2±2.7 g; $t(94)$=0.24]. However, by the test day (day 11), control rats weighed more than chronically stressed rats [stressed: 274.3±2.7 g; controls: 301.1±2.9 g; $t(94)$=6.7, P<0.01].

Systemic injection of 1.5 mg/kg DOI significantly increased rectal temperature in both stressed and control rats over time [2 days of stress: $F(4,40)$=33.5, P<0.01; 10 days of stress: $F(6,150)$=69.93, P<0.01]. This effect of DOI compared to saline injected rats was confirmed by the AUC data for rats exposed to 10 days of chronic stress [Fig. 1, $F(1,18)$=25.5 , P<0.01] and non-stressed control rats [$F(1,19)$=28.6, P<0.01].

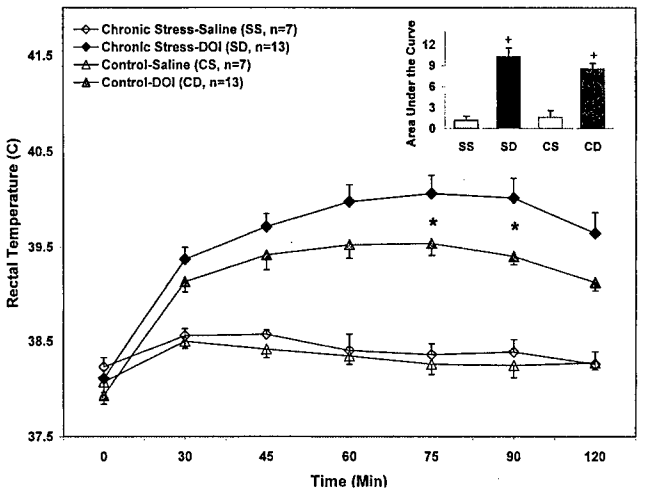

Fig. 1 Injection of 1.5 mg/kg DOI increased rectal temperature of stressed and non-stressed rats relative to basal temperature (time=0) prior to injection ($P<0.05$). Exposure to 10-day stress protocol augmented DOI-induced hyperthermia compared to non-stressed controls when measured 12 h after stress ended (*$P<0.05$ at 75 and 90 min). *Inset*: comparison of rectal temperatures between groups by AUC (sum of rectal temperature at each time point-baseline temperature). DOI increased rectal temperature in both stressed (*SD*) and control rats (*CD*) compared to saline injected stressed (*SD*) or control (*CD*) rats (+$P<0.05$)

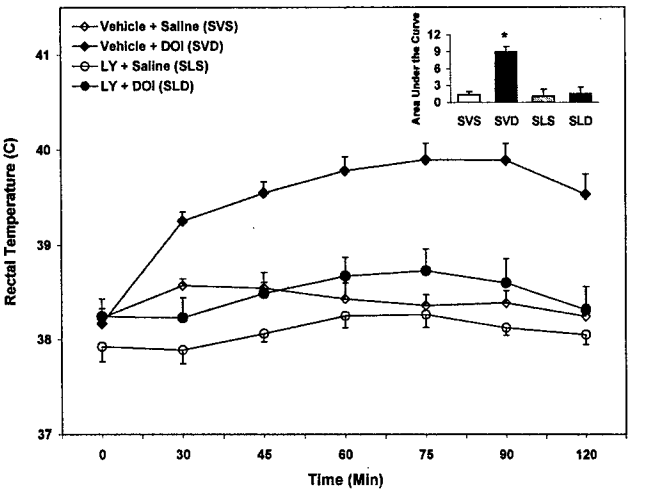

Fig. 2 Injection of 1.5 mg/kg DOI increased rectal temperature of stressed rats relative to basal temperature (time=0) prior to injection ($P<0.05$). Injection of LY-53,857 30 min prior to DOI injection attenuated the DOI-induced hyperthermia. There was no effect of LY-53,857 injection prior to saline or saline injection prior to saline. *Inset*: comparison of rectal temperatures between groups by AUC (sum of rectal temperature at each time point-baseline temperature). DOI increased rectal temperature in stressed rats pretreated with the vehicle (*SVD*) compared to rats pretreated with LY-53,587 (*SLD*) and rats injected with saline and pretreated with vehicle (*SVS*) or LY-53,587 (*SLS*) rats (*$P<0.05$)

Table 1 Effects of 1.5 mg/kg DOI (IP) on rectal temperature in non-stressed control and stressed rats. Stressed rats (n=6) were exposed to 2 days of unpredictable stress, while control rats (n=6) were weighed daily. There was no effect of the 2-day stress procedure on DOI-induced hyperthermic response. DOI increased rectal temperature in both control and stressed rats at all time points compared to pre-injection temperatures (time=0)

Time (min)	Non-stressed control	2-Day stress procedure
0	38.03±0.16	38.08±0.15
30	39.17±0.21	39.05±.019
60	39.43±0.27	39.00±0.17
90	39.32±0.29	39.03±0.10
120	38.95±0.22	38.92±0.24

Rats exposed to 10 days of chronic stress had higher rectal temperatures than control rats as indicated by a significant group by time interaction [Fig. 1, $F(6,150)=1.35$, $P<0.05$]. DOI-induced increase in rectal temperature did not differ between rats exposed to 2 days of the stress procedures and non-stressed controls [Table 1, $F(4,40)=1.37$, NS]. Therefore, the 10-day chronic stress procedure was used for subsequent experiments.

The 5-HT_2 receptor antagonist LY-53,587 attenuated the DOI-stimulated hyperthermic response of stressed [Fig. 2, $F(18,228)=7.1$, $P<0.01$) and control rats [Fig. 3, $F(18,234)=7.7$, $P<0.01$]. Both stressed and control rats injected with vehicle and DOI had significantly higher rectal temperatures than rats injected with LY-53,587 and saline, or LY-53,857 and DOI [AUC stressed: $F(3,38)=14.6$; AUC control: $F(3,39)=13.7$].

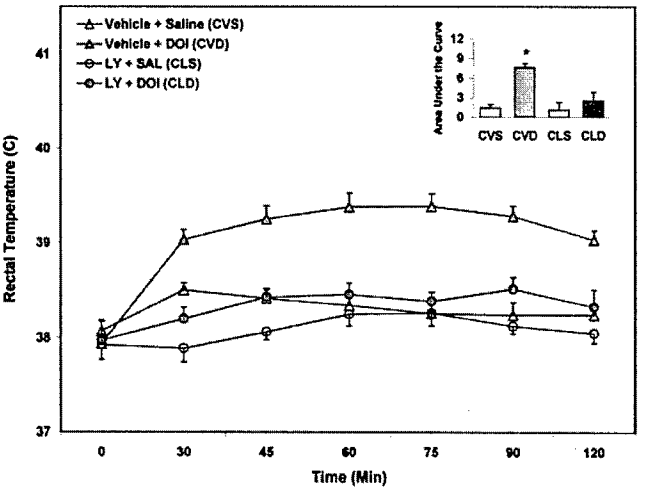

Fig. 3 Injection of 1.5 mg/kg DOI increased rectal temperature of non-stressed rats relative to basal temperature (time=0) prior to injection ($P<0.05$). Injection of LY-53,857 30 min prior to DOI injection attenuated the DOI-induced hyperthermia. There was no effect of LY-53,857 injection prior to saline or saline injection prior to saline. *Inset*: comparison of rectal temperatures between groups by AUC (sum of rectal temperature at each time point-baseline temperature). DOI increased rectal temperature in control rats pretreated with the vehicle (*CVD*) compared to rats pretreated with LY-53,587 (*CLD*) and rats injected with saline and pretreated with vehicle (*CVS*) or LY-53,587 (*CLS*) rats (*$P<0.05$)

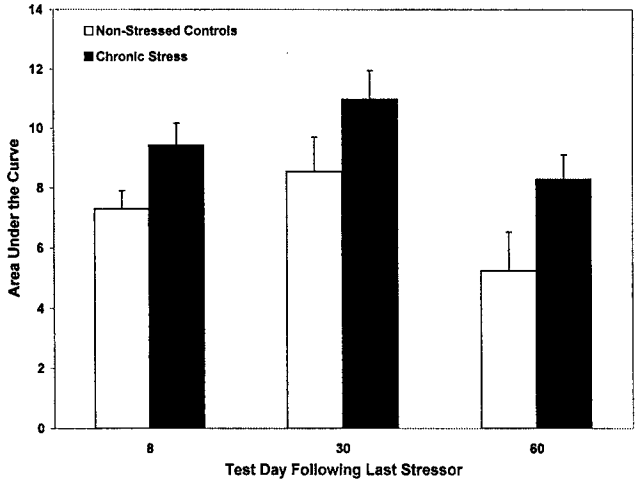

Fig. 4 Exposure to 10-day stress protocol augmented DOI-induced hyperthermia compared to non-stressed controls when measured 8, 30 or 60 days after stress ended ($P<0.05$). The rectal temperature data for five time points are summarized by AUC (sum of rectal temperature at each time point-baseline temperature)

Table 2 Effects of 1.5 mg/kg DOI (IP) on rectal temperature in non-stressed control and chronically stressed rats. DOI was injected 8, 30 or 60 days following the termination of the 10-day chronic stress protocol. Both groups showed a significant increase in rectal temperature at all time points after the drug injection (30, 60, 75, 90, and 120 min) compared to pre-injection rectal temperature (0 min). Rats exposed to 10 days of unpredictable stress showed higher rectal temperatures compared to non-stressed rats

Test day	Time (min)	Non-stressed control	Chronic stress
8 days	0	37.11±0.07	37.07±0.10
	30	38.08±0.11	38.50±0.06*
	60	38.55±0.13	38.92±0.08*
	75	38.69±0.15	39.10±0.10*
	90	38.81±0.19	39.12±0.13*
	120	38.71±0.20	38.91±0.17
30 days	0	37.44±0.22	37.58±0.16
	30	38.76±0.12	38.91±0.15
	60	38.89±0.18	39.56±0.27
	75	39.18±0.19	39.97±0.30*
	90	39.44±0.30	40.18±0.31
	120	39.46±0.46	40.26±0.29
60 days	0	37.58±0.15	37.57±0.19
	30	38.41±0.19	38.84±0.15
	60	38.50±0.22	39.13±0.17*
	75	38.59±0.59	39.26±0.15*
	90	38.77±0.27	39.41±0.17
	120	38.89±0.33	39.51±0.23

*$P<0.05$ compared to non-stressed controls

Effects of DOI 8, 15, 30 or 60 days following chronic stress

Overall, DOI significantly increased body temperature compared to pre-injection rectal temperatures on all test days [$F(5,390)=276.15$, $P<0.01$]. A similar effect was observed when each test day was analyzed separately [day 8: $F(5,125)=209.8$, $P<0.01$; day 30: $F(5,75)=96.7$, $P<0.01$; day 60: $F(5,100)=64.9$, $P<0.01$]. Stressed rats had significantly higher rectal temperatures compared to non-stressed controls on all test days [Fig. 4, AUC $F(1,60)=11.1$, $P<0.01$]. These differences between stressed and control rats were also evident when each day was analyzed separately [Table 2, day 8: $F(5,125)=3.1$, $P<0.05$; day 30: $F(5,75)=3.1$, $P=0.05$; day 60: $F(5,100)=3.6$, $P<0.05$].

Discussion

Rats exposed to 10, but not 2, days of unpredictable stress exhibited higher rectal temperatures following an injection of the 5-$HT_{2A/C}$ receptor agonist, DOI, than did non-stressed rats (Fig. 1). The DOI-induced hyperthermia was attenuated by the 5-HT_2 receptor antagonist LY-53,587, suggesting that the DOI-induced hyperthermia is mediated by 5-HT_2 receptors (Figs 2 and 3). Interestingly, the effects of chronic stress on the 5-$HT_{2A/C}$ mediated hyperthermia persisted after the termination of the stress protocol. Increases in rectal temperature following an injection of DOI continued to be greater in chronically stressed rats compared to non-stressed controls when tested 8, 30 and 60 days following the stress procedure (Fig. 4).

Stimulation of 5-HT_{2A} receptors has been shown to increase body temperature in rats (Pranzatelli and Pluchino 1990; Aulakh et al. 1994; Mazzola-Pomietto et al. 1995; Lin et al. 1998; Salmi and Ahlenius 1998). Consistent with the present study, these hyperthermic responses following DOI or other serotonergic drugs were attenuated by pretreatment with 5-HT_2 receptor antagonists, such as LY-53,587, ritanserin or ketanserin (Gudelsky et al. 1986; Mazzola-Pomietto et al. 1995; Salmi and Ahlenius 1998; Nisijima et al. 2001). Thus, the current findings and previous studies suggest that DOI stimulates an increase in body temperature through its actions at the 5-HT_2 receptor.

In agreement with the present hyperthermia findings, several studies have reported that chronic stress enhances other 5-$HT_{2A/C}$ receptor-mediated behaviors, such as wet dog and head shakes. Head twitch in mice and wet dog shakes in rats were augmented following repeated shock or psychosocial stress (Metz and Heal 1986; Gorzalka et al. 1998). These findings suggest that chronic stress enhances the number or activity of 5-$HT_{2A/C}$ receptors. Metz and Heal (1986) reported an increase in cortical 5-HT_2 receptor binding following repeated shock treatment, as their head twitch data would suggest. Other studies have found increases in 5-HT_{2A} receptors following exposure to repeated stress (Torda et al. 1988, 1990; Davis et al. 1995; McKittrick et al. 1995; Takao et al. 1995; Berton et al. 1998; Ossowska et al. 2001). Therefore, an increase in the affinity or density of 5-$HT_{2A/C}$ receptors produced by 10 days of unpredictable stress may account for the potentiated hyperthermic response observed following the injection of DOI.

The augmented hyperthermia in rats exposed to 10 days of unpredictable stress persisted for 60 days after the stress procedure ended. Given the above discussion, this suggests that stress-induced alterations in the 5-$HT_{2A/C}$ receptor system are long-lasting, even in the absence of additional stressors. Several studies have reported lasting behavioral changes following various chronic or acute stress exposures, such as enhanced fear responses to a novel environment (Desan et al. 1988; Van Dijken et al. 1992a, 1992b; Adamec and Shallow 1993; Pynoos et al. 1996; Koba et al. 2001), escape deficit (Mangiavacchi et al. 2001) or increased self-administration of amphetamine or cocaine (Piazza et al. 1990; Covington and Miczek 2001). To our knowledge, this is the first study that has found a persistent physiological change following exposure to stress that can be attributed to the 5-HT system, specifically.

The brain regions involved in the stress-induced 5-$HT_{2A/C}$ receptor alterations that are responsible for the persistent hyperthermia are unknown; however, an increase in 5-HT_{2A} receptor density in cortex following exposure to stress has been reported (Metz and Heal 1986; Torda et al. 1988, 1990; Davis et al. 1995; McKittrick et al. 1995; Takao et al. 1995; Berton et al. 1998; Ossowska et al. 2001). Alternatively, 5-HT receptors in the hypothalamus are known to mediate temperature regulation (Lin et al. 1983, 1998; Lin and Pivorun 1986) and may also account for the present findings. An injection of DOI (0.2 μg) into the anterior hypothalamus/preoptic area increased colonic temperature, similar to the systemic administration (Lin et al. 1998). Likewise, elevating 5-HT concentrations in the hypothalamus increased colonic temperature, thus, supporting the relationship between 5-HT, the hypothalamus and temperature regulation (Lin et al. 1998).

Several mechanisms may contribute to the 5-$HT_{2A/C}$ receptor alterations following exposure to chronic stress. Chronic unpredictable stress protocols, as used in the current study, have been shown to increase basal or drug-stimulated corticosterone levels in rats (Sapolsky et al. 1984; Haile et al. 2001). Corticosterone, in turn, increases the density of 5-$HT_{2A/C}$ receptors and facilitates 5-HT_2 mediated behaviors (Kuroda et al. 1992; McKittrick et al. 1995; Berendesen et al. 1996; Fernandes et al. 1997; Gorzalka et al. 2001). The corticosterone-stimulated increase in 5-HT_2 mediated behaviors can be attenuated by the administration of a 5-HT_{2A} receptor antagonist (Gorzalka et al. 2001). It remains to be determined if administration of a 5-HT_2 receptor antagonist or a glucocorticoid receptor antagonist during the chronic stress procedure would block the stress-induced hyperthermia.

Alterations in 5-HT transmission have been recorded in many brain regions during and following physical and social stress. Exposure to chronic social stress increases the metabolism of 5-HT in several limbic brain regions, including the preoptic area of the hypothalamus (Blanchard et al. 1991; Berton et al. 1998). Likewise, extracellular levels of 5-HT in the cortex or hypothalamus are elevated during acute immobilization stress (for review see Shimizu et al. 1992; Kawahara et al. 1993; Chaouloff et al. 1999; Matuszewich et al. 2002). Although chronic changes in 5-HT release may alter the density of 5-HT receptors, 5,7-dihydroxytryptamine lesions do not appear to alter the effects of acute immobilization stress on cortical 5-HT_{2A} receptors or 5-HT_{2A} mediated behavior (Torda et al. 1990; Yamada et al. 1995). Despite the fact that acute stress increases 5-HT release, it remains to be determined whether chronic stress produces persistent changes in 5-HT release, which could account for the observed 5-HT-mediated hyperthermic responses. Alternatively, other neurotransmitters or hormones influenced by chronic stress may regulate 5-HT receptor responsiveness (Torda et al. 1990; Gorzalka et al. 2001).

The observed alterations in 5-HT-mediated temperature regulation following chronic, unpredictable stress may be specific to the types of stressors applied or the parameters of the stress exposure. For example, while acute immobilization stress increased extracellular 5-HT in the prefrontal cortex (Kawahara et al. 1993; Matuszewich et al. 2002), forced swim stress for 5 min decreased 5-HT levels (Adell et al. 1997) and did not change 5-HT levels when rats swam for 30 min (Kirby et al. 1995). In the current protocol, a variety of stressors are employed, including swim stress and immobilization. It is not known whether a particular stressor was more critical for changing the 5-HT receptor systems in the current procedure. Sixteen days of exposure to chronic unpredictable stressors, similar to the procedure and types of stressors used in the current study, increased 5-HT_{2A} receptors in the cortex (Ossowska et al. 2001), although the particular stressors used in the protocol have yielded different effects when applied individually (Torda et al. 1988; Chaouloff et al. 1994; Okuyama et al. 1995; Yamada et al. 1995; Durand et al. 1998). In the current study, 2 days of unpredictable stress exposure was insufficient to augment 5-$HT_{2A/C}$-mediated hyperthermic responses (Table 1), suggesting that more than 2 days of unpredictable stressors may be necessary for the 5-HT2A/C mediated behavior changes.

Chronic, unpredictable stress in rodents produces persistent changes in 5-$HT_{2A/C}$ receptor-mediated hyperthermia that last as long as 60 days following the termination of the last stressor. These long-term changes in 5-HT receptor responsiveness parallel the cortical 5-HT_{2A} receptor changes observed in patients with depression (Stahl 1994) and in humans who committed suicide (for review, see Ferrier et al. 1986; McKeith et al. 1987; Arora and Meltzer 1989; Arango et al. 1995; Mann 1998). Thus, the chronic, unpredictable stress model may be useful in identifying neurochemical changes associated with human disorders, such as depression or post-traumatic stress disorder.

Acknowledgements This work was supported by National Institutes of Health grants DA07606 and DA05937-02 and by Department of Defense grant 17-99-1-9479.

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APPENDIX 3

Behavioral/Systems/Cognitive

High-Dose Methamphetamine Acutely Activates the Striatonigral Pathway to Increase Striatal Glutamate and Mediate Long-Term Dopamine Toxicity

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Methamphetamine (METH) has been shown to increase the extracellular concentrations of both dopamine (DA) and glutamate (GLU) in the striatum. Dopamine, glutamate, or their combined effects have been hypothesized to mediate striatal DA nerve terminal damage. Although it is known that METH releases DA via reverse transport, it is not known how METH increases the release of GLU. We hypothesized that METH increases GLU indirectly via activation of the basal ganglia output pathways. METH increased striatonigral GABAergic transmission, as evidenced by increased striatal GAD65 mRNA expression and extracellular GABA concentrations in substantia nigra pars reticulata (SNr). The METH-induced increase in nigral extracellular GABA concentrations was D1 receptor-dependent because intranigral perfusion of the D1 DA antagonist SCH23390 (10 μM) attenuated the METH-induced increase in GABA release in the SNr. Additionally, METH decreased extracellular GABA concentrations in the ventromedial thalamus (VM). Intranigral perfusion of the GABA-A receptor antagonist, bicuculline (10 μM), blocked the METH-induced decrease in extracellular GABA in the VM and the METH-induced increase in striatal GLU. Intranigral perfusion of either a DA D1 or GABA-A receptor antagonist during the systemic administrations of METH attenuated the striatal DA depletions when measured 1 week later. These results show that METH enhances D1-mediated striatonigral GABAergic transmission (1), which in turn activates GABA-A receptors in the SNr (2), leading to a decrease in GABAergic nigrothalamic activity (3), an increase in corticostriatal GLU release (4), and a consequent long-term depletion of striatal DA content (5).

Key words: GABA; D1; GABA-A; substantia nigra; thalamus; microdialysis

Introduction

The abuse of the psychostimulant methamphetamine (METH) has grown over the last decade. One major concern of METH abuse is the potential long-term striatal dopaminergic and serotonergic deficits associated with repeated exposure over time. Specifically, studies in rodents and primates show long-term decreases in markers associated with dopamine (DA) and serotonin (5-HT) toxicity, including decreases in monoamine transporter immunoreactivities, tyrosine and tryptophan hydroxylase activities, and terminal degradation (Hotchkiss and Gibb, 1980; Wagner et al., 1980; Ricaurte et al., 1982).

METH acutely increases extracellular concentrations of DA and glutamate (GLU) in the striatum (O'Dell et al., 1991; Stephans and Yamamoto, 1994). The combined effect of DA and GLU release is thought to produce oxidative stress and glutamate-mediated excitoxicity to DA nerve terminals (Sonsalla et al., 1989; Nash and Yamamoto, 1992; Yamamoto and Zhu, 1998; LaVoie and Hastings, 1999; Imam et al., 2001). Although METH is known to release DA directly via reverse transport, little is known about the mechanisms of METH-induced GLU release in the striatum.

The glutamatergic innervation of the striatum arises primarily from corticostriatal terminals (Gerfen, 1989; Bellomo et al., 1998). The corticostriatal pathway can be regulated by the output pathways of the basal ganglia involving the nigrothalamic GABAergic and thalamocortical glutamatergic projections (Kaneko and Mizuno, 1988). Specifically, GABAergic projection neurons from the striatum terminate mainly in the substantia nigra pars reticulata (SNr). The SNr contains a high density of DA D1 receptors, mainly distributed on presynaptic striatonigral terminals (Altar and Hauser, 1987; Martin and Waszczak, 1994; Trevitt et al., 2002) to positively modulate GABA release within the SNr (Reubi et al., 1977, 1978; Kelly et al., 1985; Aceves et al., 1995). This dopaminergic regulation of GABAergic neurotransmission within the SNr can directly influence the nigrothalamic pathway via postsynaptic GABA-A receptors distributed on the soma of nigrothalamic projections (Nicholson et al., 1995) and consequently affect thalamocortical activity (Timmerman and Westerink, 1997). Therefore, activation of SNr GABA-A receptors via METH-induced increases in DA release and D1-

Received Sept. 1, 2004; revised Nov. 12, 2004; accepted Nov. 13, 2004.

This work was supported by National Institutes of Health Grant DA07606, Department of the Army Grant DAMD 17-99-1-9479, and a gift from Hitachi America.

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DOI:10.1523/JNEUROSCI.3597-04.2004

 0270-6474/04/2411449-08$15.00/0

mediated stimulation of GABA release by METH could disinhibit thalamocortical glutamatergic activity and increase corticostriatal GLU release.

The overarching hypothesis of this study is that METH triggers a polysynaptic process characterized by (1) enhanced striatonigral GABAergic transmission, (2) activation of GABA-A receptors in the SNr leading to decreased nigrothalamic activity, (3) disinhibition of thalamocortical activity, and ultimately, (4) increased corticostriatal GLU release and (5) long-term depletion of striatal DA content. To test this hypothesis, we examined the effects of METH on GAD65 mRNA expression in the striatum and extracellular GABA concentrations in the SNr and ventromedial thalamus (VM). Moreover, because DA D1 receptors positively modulate GABA release within the SNr, and GABA-A receptors are distributed on nigrothalamic projections, we examined whether an antagonist to D1 or GABA-A receptors into the SNr would block the hypothesized decreases in extracellular GABA within the VM, the increase in extracellular GLU in the striatum, and the long-term decrease in DA content within the striatum.

Materials and Methods

Subjects

Male Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) weighing 175–200 gm at the beginning of experimental procedures were housed under a 12 hr light/dark cycle (lights on from 7:00 A.M. to 7:00 P.M.) in a temperature-controlled (21–23°C) and humidity-controlled room. The rats were initially housed four per cage until the day of surgery, on which they were then individually housed. Food and water were available *ad libitum*. All experimental procedures were performed between 7:00 A.M. and 7:00 P.M. and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drugs

1(*S*),9(*R*)-(−)-Bicuculline methobromide (BIC) and *R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH 23390) were obtained from Sigma-Aldrich (St. Louis, MO). METH was supplied by the National Institute on Drug and Abuse (Research Triangle Park, NC). Doses of METH refer to the weight of the salt. Four injections of METH (10 mg/kg) were administered intraperitoneally every 2 hr. BIC (10 μM) and SCH 23390 (10 μM) were administered via reverse dialysis in modified Dulbecco's buffered saline (in mM: 137 NaCl, 2.7 KCl, 0.5 $MgCl_2$, 8.1 Na_2HPO_4, 1.5 KH_2PO_4, 1.2 $CaCl_2$, and 5 D-glucose, pH 7.4). SCH 23390 (4 mg) was initially dissolved in 50 μl of glacial acetic acid followed by 950 ml of Dulbecco's buffered saline to produce a stock solution. This stock solution was diluted to 10 μM with Dulbecco's buffer, and pH was adjusted to 7.4 with 0.1 N NaOH. The vehicle control for this perfusion medium was prepared identically but without the SCH 23390.

Experimental procedures

For the microdialysis experiments, all rats were anesthetized with a combination of xylazine (12 mg/kg) and ketamine (80 mg/kg) and placed into a Kopf stereotaxic frame. The skull was exposed, and holes were drilled through the skull above the lateral striatum [anteroposterior (AP), +1.2; mediolateral (ML), ±3.2; dorsoventral (DV), −8.2 mm], ventromedial thalamus (AP, −2.3; ML, ±1.4; DV, −8.4 mm), and substantia nigra pars reticulata at a 15° angle (AP, −5.6; ML, ±4.1; DV, −10.2 mm) (Paxinos and Watson, 1982). When two probes were implanted, they were on the same side of the brain. One probe was placed in the SNr, and a second probe was placed in either the ipsilateral VM or the ipsilateral striatum. All dialysis probes were of a concentric flow design and constructed as previously described by Yamamoto and Pehek (1990). The lengths of the active dialysis membrane [Spectrapor, 6000 molecular weight cutoff, 210 μM optical density (OD)] were as follows: striatum 4 mm; thalamus 1 mm; substantia nigra 1.5 mm. The probes were then lowered to the appropriate position and secured to the skull with three stainless steel screws and cranioplastic cement.

Microdialysis procedures

The day after surgery, modified Dulbecco's phosphate buffered saline medium was pumped via a dual channel swivel (Instech Laboratories, Plymouth Meeting, PA) through the microdialysis probes with a Harvard Apparatus (Hollison, MA) model 22 syringe infusion pump at a rate of 2 μl/min, as previously described in Matuszewich and Yamamoto (1999). There was a 1.5 hr equilibration period before baseline sample collections. Baseline dialysate samples were collected for 2 hr after which the perfusion medium of the probe inserted in the SNr was switched to a medium containing either BIC, SCH 23390, or the vehicle perfusion Dulbecco's medium, pH 7.4. METH or saline was injected after the last baseline sample was collected. Subsequent dialysis samples were collected every hr for 8 hr in the initial experiments to measure GABA within the SNr during the perfusion of SCH 23390. To enhance the temporal resolution for the detection of possible changes in GABA or glutamate, all subsequent microdialysis experiments in the SNr, ipsilateral ventromedial thalamus, and ipsilateral striatum used sample collections every 30 min. The dead volumes of all probes were calculated so as to synchronize the timing and initiation of drug perfusion for the dual probe microdialysis experiments.

Rectal temperatures were measured 1 hr after each of four injections of either saline or METH.

Biochemical measurements

GABA. The concentrations of GABA in dialysate samples were determined by HPLC with electrochemical detection, as previously described by Smith and Sharp (1994). GABA was derivatized with *O*-phthaldialdehyde (OPA) and sodium sulfite. Briefly, 2 μl of the stock derivatization reagent containing 22 mg OPA, 9 ml of 0.4 M boric acid, pH 10.4, 0.5 ml of 100% ethanol, and 0.5 ml of 1 M sodium sulfite was added to 20 μl of dialysate or standard, vortexed, and allowed to react for 5 min before injecting onto a C18 column (100 × 2.0 mm, 3 μm particle size; Phenomenex, Torrance, CA). Separation of GABA was achieved with a mobile phase consisting of 0.1 M sodium phosphate and 0.1 mM EDTA in 10% methanol at pH 4.4. GABA was detected with an LC-4B amperometric detector (Bioanalytical Systems, Inc., Lafayette, IN) using a 6 mm glassy working electrode maintained at a potential of 0.7 V relative to an Ag–AgCl reference electrode.

Glutamate. The concentrations of GLU in dialysate samples were determined by HPLC coupled to fluorescence detection. GLU was derivatized with OPA (Donzanti and Yamamoto, 1988). Briefly, the stock derivatization reagent was prepared by dissolving 27 mg OPA in 9 ml of 0.1 M sodium tetraborate, pH 9.4, and 1 ml of 100% methanol to which 15 μl β-mercaptoethanol was added. This stock solution was then diluted 1:3 with sodium tetraborate buffer. A 10 μl aliquot of this reagent solution was added to 20 μl of dialysate or standard, vortexed, and allowed to react for 1.5 min before injecting onto a C18 column (100 × 2.0 mm, 3 μm particle size; Phenomenex). GLU was eluted using a mobile phase consisting of 0.1 M sodium phosphate and 0.1 mM EDTA in 10% methanol, pH 6.7. GLU was detected using a Waters 474 Scanning Fluorescence Detector (Milford, MA) with an excitation wavelength (Exλ) = 340 nm and emission wavelength (Emλ) = 440 nm.

In situ *hybridization*

Separate groups of rats were used for these studies. METH or saline was injected as described above. Five hours after the last injection, rats were killed by rapid decapitation, and whole brains were immediately frozen on dry ice. Sections from the striatum were processed for *in situ* hybridization histochemistry with riboprobes as previously described by (Nielsen and Soghomonian, 2004). Briefly, 10-μm-thick cryostat-cut frozen sagittal sections at the striatal level were produced from saline and METH-treated rats. Sections were quickly dried and fixed in 3.5% paraformaldehyde followed by prehybridization washes in triethanolamine and tris-glycine. Sections were then hybridized for 4 hr at 52°C with 4.0 ng in 20 μl of radiolabeled cRNA probe per section (average specific activity: 4.3×10^5 cpm/ng). The [^{35}S]-labeled cRNA probe was synthesized from a rat GAD65 cDNA inserted into bluescript SK plasmid, which was linearized with *Hind*III. The cRNA probe was diluted in hybridiza-

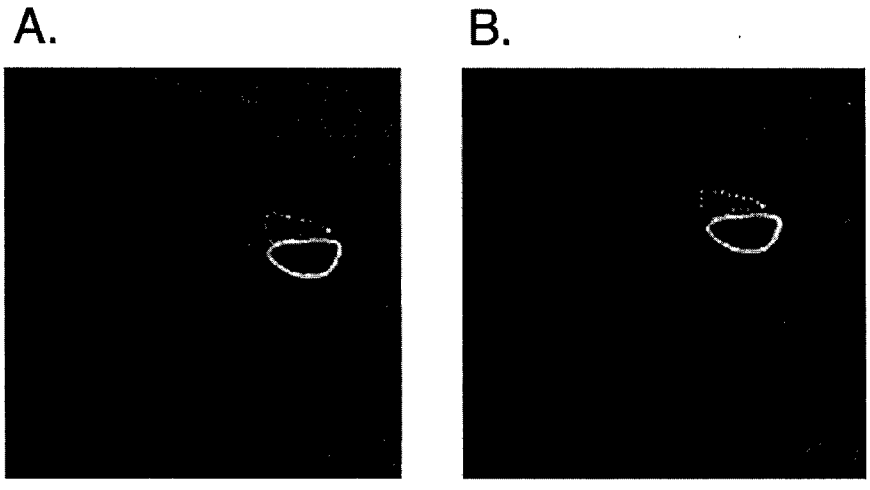

Figure 1. *A, B,* Negative images of x-ray films from sagittal brain sections processed for *in situ* hybridization with a ^{35}S-labeled cRNA probe for GAD65 mRNA. *A,* Saline-control and (*B*) METH-treated rats. The division of the dorsal and ventral neostriatum is noted by the dotted areas. The quantitative analysis of labeling was performed in the ventral and dorsal neostriatum (denoted by the white dotted areas).

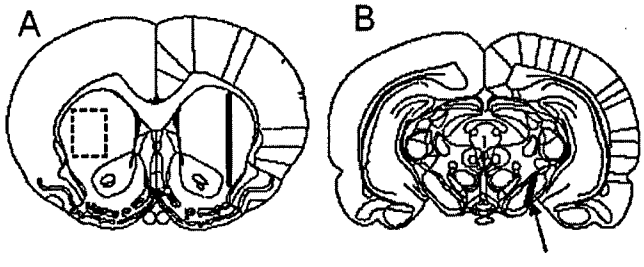

Figure 2. *A, B,* Schematic diagram of probe placement in the lateral striatum (*A*) and the substantia nigra pars reticulata (*B*). The regions were (in millimeters relative to bregma): lateral striatum (AP +1.2) and substantia nigra pars reticulata (AP −5.6). The black lines represent the probe membrane in the region. The arrow points to the location of the probe in the substantia nigra. The hatched box on the left side of the diagram in *A* represents the area within the striatum dissected for DA tissue content analysis.

tion solution containing 40% formamide, 10% dextran sulfate, 1× SSC, 10 mM dithiothreitol, 1.0% sheared salmon sperm DNA, 1.0% yeast tRNA, and 1× Denhardt. The sections were then subsequently washed in 50% formamide at 52°C for 5 and 20 min, RNase A (100 μg/ml; Sigma-Aldrich) for 30 min at 37°C, and in 50% formamide for 5 min at 52°C. Sections were then dehydrated in ethanol and defatted in xylene and apposed to Kodak Biomax-MR x-ray films, exposed in light-tight cassettes for 11–14 d. Films were developed in Kodak D-19 for 3.5 min at 14°C.

Quantification of labeling

Levels of GAD65 mRNA labeling in the dorsal and ventral neostriatum were quantified on x-ray films by computerized densitometry with a Macintosh computer, a Sony CCD video camera, and the NIH Image software. The OD of labeling in the striatum was calculated after subtracting the optical density of the film and standardization against emulsion-coated filters (Eastman Kodak, Rochester, NY). The value for each rat was calculated as the average value from three sections. The average level of labeling was then calculated for rats injected with saline or METH.

Tissue content analysis

Seven days after dialysis, all rats were killed by rapid decapitation, and brains were removed and quick-frozen in dry ice. Brains were sectioned on a cryostat (−20°C). The striatum on the side ipsilateral and contralateral to the probe placements in the SNr and VM was dissected from 400 μm coronal sections. Tissue samples were sonicated in 300 μl of cold 0.1N $HClO_4$ and centrifuged at 14,000 × *g* for 6 min at 4°C. DA was separated on a C18 column (100 × 2.0 mm, 3 μm particle size; Phenomenex) and eluted with a mobile consisting of 32 mM citric acid, 54.3 mM sodium acetate, 0.074 mM EDTA (Na_2-EDTA), 0.215 mM octyl sodium sulfate, and 3% methanol, pH 3.8. Separation of DA and DOPAC was confirmed before each dialysis experiment. Compounds were detected with an LC-4C amperometric detector (Bioanalytical Systems, West Lafayette, IN) with a glassy carbon working electrode maintained at a potential of +0.670 V relative to an Ag–AgCI reference electrode. Data were recorded using the EZ Chrom (Scientific Software, Pleasanton, CA) software package. Concentrations were expressed as picogram per microgram of protein. Protein content was determined by method of Bradford.

Histology

All dialysis probe placements in the striatum, SNr, and VM were verified from 40 μm coronal sections. Only data from experiments with verified probe placements were included in the statistical analysis.

Statistical analysis

Striatal DA tissue content was analyzed using two-way ANOVA followed by Tukey's *post hoc* test to determine significant differences between treatment groups. For the dialysis experiments, all data are presented as percentage of baseline to standardize across all the treatment groups and allow for an effective comparison between conditions for GLU and GABA. Changes in amino acid concentrations over time as a function of treatment were analyzed by a two-way ANOVA with repeated measures, using treatment as a between subjects variable and time as a repeated measures variable. For the *in situ* hybridization studies, optical density measures between two groups were statistically analyzed by a Student's *t* test. In all cases, a level of $p < 0.05$ was considered statistically significant.

Results

In situ hybridization was used to assess METH-induced changes in striatal GAD65 mRNA levels. Figure 1 illustrates GAD65 mRNA levels as visualized on x-ray films in a saline-treated (Fig. 1*A*) and a METH-treated rat (Fig. 1*B*). High-dose METH significantly increased GAD65 mRNA levels by 35.6 and 29.7% in the ventral and dorsal neostriatum, respectively, of rats killed 5 hr after METH compared with saline-treated rats [ventral neostriatum: METH, 0.1440 ± 0.007; saline, 0.106 ± 0.006 (mean relative OD ± SEM), METH vs saline; $T = 3.92$; $p < 0.05$; dorsal neostriatum: METH, 0.135 ± 0.005; saline, 0.104 ± 0.007 (mean relative OD ± SEM), METH vs saline; $T = 3.35$; $p < 0.05$]. There was no significant difference in METH-induced increases in GAD65mRNA expression between the dorsal and ventral regions of the neostriatum.

In vivo microdialysis experiments were performed to investigate the effects of METH on the GABAergic striatonigral pathway. Figure 2, *A* and *B*, illustrates the locations of the microdialysis probes. Figure 3 illustrates the effect of high-dose METH on the basal extracellular concentrations of GABA in the SNr. The basal concentration of GABA in dialysate from the SNr was 26.2 ± 3.4 pg/20 μl. METH significantly increased extracellular concentrations of GABA in the SNr by 50%. Perfusion of the D1 antagonist SCH 23390 (10 μM) in the SNr attenuated the METH-induced increases in extracellular GABA. Intranigral perfusion of SCH 23390 alone did not significantly affect basal extracellular concentrations of GABA in the SNr (Fig. 3).

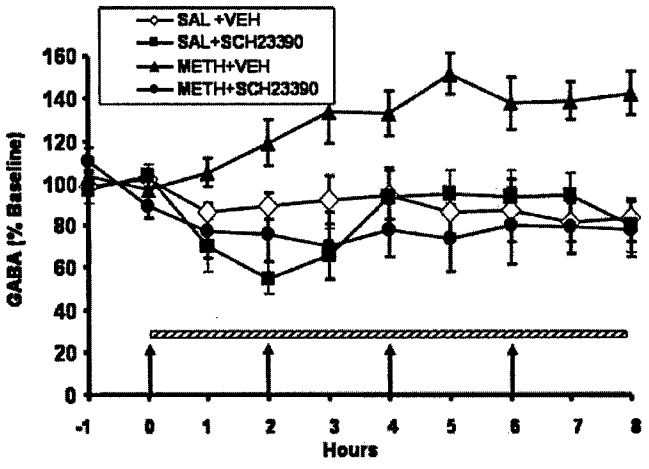

Figure 3. The effects of intranigral perfusion of SCH23390 (10 μM) on METH-induced changes in extracellular GABA concentrations in the SNr. Four injections of METH (10 mg/kg) or 0.9% saline (1 ml/kg) were given every 2 hr (indicated by arrows). Dialysate samples were collected in the SNr every hour during the 2 hr baseline period and for the 8 hr drug treatment period. SCH23390 was reverse dialyzed into the SNr after the baseline period and continuously perfused until the end of the experiment (indicated by hatched bar). Data are expressed as mean ± SEM percentage of baseline values. METH plus vehicle treatment group had a significant increase in extracellular GABA concentrations compared with METH plus SCH23390 (two-way ANOVA with repeated measures, SCH23390 simple main effect; $F_{(1,72)} = 5.17$; $p < 0.05$). There is no difference between saline plus vehicle-, saline plus SCH23390-, and METH plus SCH23390-treated rats. $n = 8–9$ rats per group.

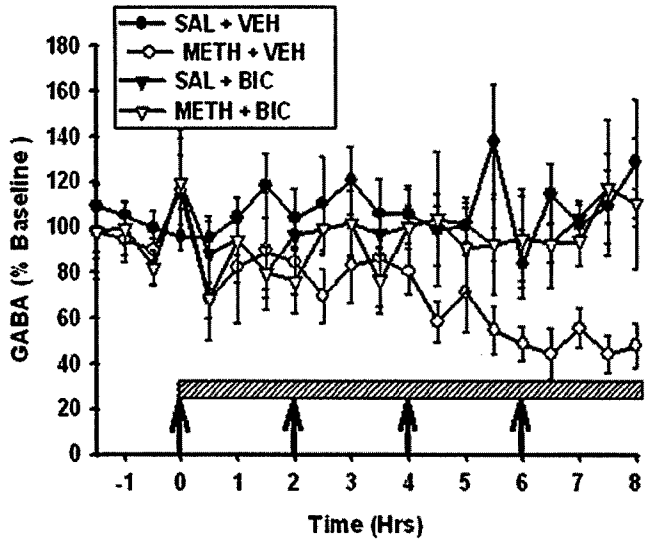

Figure 4. The effects of the intranigral perfusion of the GABA-A receptor antagonist BIC (10 μM) on METH-induced changes in extracellular GABA in the VM thalamus. Four injections of METH (10 mg/kg) or 0.9% saline (1 ml/kg) were given every 2 hr (indicated by arrows). Dialysate samples were collected in the VM every hour during the 2 hr baseline period and the 8 hr drug treatment period. BIC was reverse dialyzed into the SNr after the baseline period and continuously perfused until the end of the experiment (indicated by hatched bar). Data are expressed as mean ± SEM percentage of baseline values. METH plus vehicle group showed a significant decrease in extracellular GABA concentrations compared with METH plus BIC (two-way ANOVA with repeated measures, BIC main effect; $F_{(1,379)} = 15.34$; $p < 0.05$). There was no difference between saline plus vehicle-, saline plus BIC-, and METH plus BIC-treated rats. $n = 9–10$ rats per group.

A separate series of experiments was performed to investigate the effect of METH on basal extracellular concentrations of GABA in the VM. The basal concentration of GABA in dialysate from the VM was 25.6 ± 2.4 pg/20 μl. Figure 4 illustrates the effect of METH on extracellular concentrations of GABA in the

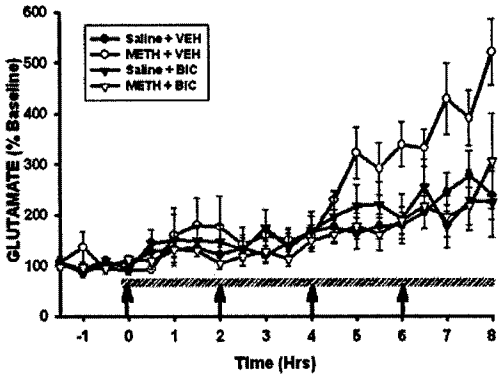

Figure 5. The effects of intranigral perfusion of bicuculline on METH-induced GLU release in the striatum. Four injections of METH (10 mg/kg) or 0.9% saline (1 ml/kg) were given every 2 hr (indicated by arrows). Dialysate samples were collected in the striatum every 30 min during the 2 hr baseline period and the 8 hr drug treatment period. BIC was reverse dialyzed into the SNr after the baseline period and continuously perfused until the end of the experiment (indicated by hatched bar). Data are expressed as mean ± SEM percentage of baseline values. METH plus vehicle group showed a significant increase in extracellular GLU concentrations compared with METH plus BIC (two-way ANOVA with repeated measures; BIC main effect; $F_{(1,339)} = 7.764$; $p < 0.05$). There was no difference between saline plus vehicle-, saline plus BIC-, and METH plus BIC-treated rats. $n = 8–9$ rats per group.

VM. METH significantly decreased extracellular concentrations of GABA in the VM by 35%. This decrease in extracellular GABA occurred after the third METH injection and was sustained up to 5 hr after the last injection (data not shown). Intranigral perfusion of the GABA-A receptor antagonist bicuculline BIC (10 μM) into the SNr significantly attenuated the METH-induced decrease in extracellular GABA concentrations in the ipsilateral VM (Fig. 4). Intranigral perfusion of BIC alone did not have a significant effect on basal concentrations of extracellular GABA in the ipsilateral VM.

Figure 5 illustrates the effect of intranigral perfusion of BIC on METH-induced increases in extracellular GLU concentrations in the ipsilateral striatum. The basal concentration of GLU in dialysate from the striatum was 1211 ± 3 pg/20 μl. METH produced a gradual and significantly sustained increase in extracellular GLU concentrations in the lateral striatum. Perfusion of BIC into the SNr significantly attenuated the METH-induced increases in extracellular concentrations of GLU in the ipsilateral striatum (Fig. 5). Intranigral perfusion of BIC alone did not have a significant effect on basal concentrations of extracellular GLU in the ipsilateral striatum.

Figure 6 illustrates the effect of perfusion of SCH 23390 in the SNr on striatal tissue concentrations of DA 7 d after systemic METH administration. METH produced a significant depletion of DA content in striatal tissue. Intranigral perfusion of SCH 23390 during the administration of METH significantly attenuated the METH-induced depletion of DA content measured 7 d later in the side ipsilateral to the side of the intranigral perfusion.

Figure 7 illustrates the effect of intranigral perfusion of BIC on striatal tissue concentrations of DA 7 d after systemic METH administration. METH produced significant depletions in DA tissue content in the striatum contralateral to the side of the intranigral perfusion. In contrast, the perfusion of BIC into the SNr during the systemic administration of METH completely blocked the long-term METH-induced DA depletions in the striatum ipsilateral to the intranigral perfusion of BIC when measured 7 d after the microdialysis experiments. Intranigral perfusion of BIC alone did not have a significant effect on DA tissue

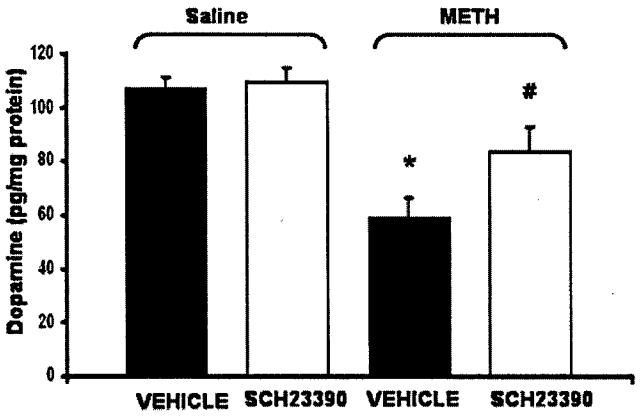

Figure 6. The effect of the intranigral perfusion of the D1 antagonist SCH23390 on long-term METH-induced striatal DA depletions. Rats were killed 1 week after microdialysis. Data are expressed as mean ± SEM picogram per milligram of protein. Striatal DA content of METH-treated rats was significantly less compared with saline-treated rats ($F_{(1,103)} = 5.23; p < 0.05$). There was a significant interaction between saline- and METH-administered VEH or SCH23390 ($F_{(1,103)} = 4.03; p < 0.05$). Intranigral perfusion of SCH23390 significantly attenuated the METH-induced depletions of DA ($p < 0.05$) (METH plus SCH23390 vs METH plus VEH; $T = 2.08; p < 0.05$) but did not block the effects of METH because METH plus SCH23390 was still significantly different from saline plus VEH ($T = 2.90; p < 0.05$). There was no difference between saline plus vehicle and saline plus SCH23390-treated rats. * indicates significant ($p < 0.05$) difference from METH plus vehicle and saline plus VEH-treated rats. # indicates significant ($p < 0.05$) difference from METH plus vehicle and METH plus SCH23390. $n =$ 6–7 rats per group.

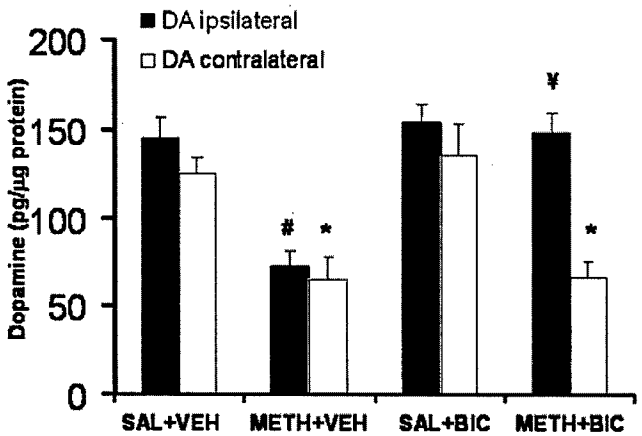

Figure 7. The effects of the intranigral perfusion of BIC on METH-induced striatal DA depletions. Rats were killed 1 week after microdialysis. Data are expressed as mean ± SEM picogram per milligram of protein. For ipsilateral side: there was a significant main effect of METH ($F_{(1,44)} = 12.823; p < 0.05$), BIC ($F_{(1,44)} = 15.592; p < 0.05$), and a significant interaction between METH and BIC ($F_{(1,44)} = 9.845; p < 0.05$). Striatal DA content of METH plus vehicle-treated rats was significantly less compared with saline-treated rats ($q = 6.82; p < 0.05$). ¥ indicates METH plus BIC was higher than METH plus VEH ($q = 6.59; p < 0.05$). # indicates significant difference ($p < 0.05$) between METH plus vehicle-, saline plus vehicle-, and saline plus BIC-treated rats. For contralateral side: there was a significant main effect of METH ($F_{(1,23)} = 75.855; p < 0.05$). Striatal DA tissue content of METH-treated rats was significantly less compared with saline plus vehicle-treated rats. * indicates significant difference in striatal DA tissue content between SAL plus BIC and METH plus BIC ($q = 5.98; p < 0.05$) and between SAL plus VEH and METH plus VEH ($q = 4.67; p < 0.05$). $n = 6–8$ rats per group.

content in the ipsilateral striatum of rats administered systemic saline injections (Fig. 7).

Rectal temperatures were measured at 1 hr after each saline or METH administration. Saline-injected controls had average rectal temperatures of 37.2 ± 0.2°C after each of four injections. The average rectal temperatures of METH-treated rats when measured an hour after each of the four injections was 39.6 ± 0.4°C. Intranigral perfusion of BIC or SCH 23390 in combination with the systemic administration of METH did not affect the hyperthermic rectal temperatures of METH treated rats when measured 1 hr after each of the METH administrations (39.7 ± 0.2°C).

Discussion

Several studies have focused on the roles of DA and GLU in mediating the neurotoxicity of METH to DA nerve terminals, but few studies have examined the possible coordinated interaction between DA and GLU within the basal ganglia circuitry that could explain the long-term depletion of striatal DA content after METH. Although some attention has been directed to the effects of high doses of METH on the striatonigral pathway (Chapman et al., 2001; Hanson et al., 2002; Johnson-Davis et al., 2002), there has been less focus on how activation of this pathway may trigger polysynaptic events culminating in increased extracellular GLU in the striatum. This study elucidates a mechanism of METH-induced GLU release in the striatum that is dependent on both dopaminergic and GABAergic transmission within the striatonigral pathway of the basal ganglia. The findings indicate that METH increases striatonigral GABAergic transmission, as evidenced by GAD65 mRNA expression in the striatum, D1-dependent increases in extracellular GABA in the SNr, and subsequent GABA-A receptor-dependent decreases in GABA release in the thalamus and increases in striatal GLU.

The METH-induced increases in striatal GAD65 mRNA expression (Fig. 1) and extracellular GABA within the SNr (Fig. 3) appear to be mediated by DA within these respective brain regions. GAD65 mRNA expression was used as an index of GABAergic activity within the striatonigral pathway. To our knowledge, this is the first report of specific changes in striatal GAD65 mRNA expression after METH. Although we cannot conclude that increases in striatal GAD65 directly translate into increases in GABA release in the SNr, the increases in striatal GAD65 mRNA may reflect long-term changes in GABAergic activity within the striatonigral pathway after METH. GAD65 gene expression in striatonigral neurons is increased by D1 activation (Laprade and Soghomonian, 1995, 1997). Therefore, METH-induced striatal DA release (Stephans and Yamamoto, 1994) presumably activates striatal D1 receptors to increase GAD65 mRNA expression. This interpretation is consistent with findings that high doses of METH preferentially affect markers of the D1 receptor-associated striatonigral path, as evidenced by changes in striatal preprodynorphin, preproenkaphalin (Wang and McGinty, 1996), and preprotachykinin mRNA (Chapman et al., 2001). Because previous work showed that METH decreases markers of toxicity to DA terminals to a greater extent in the ventral neostriatum (Eisch et al., 1992), a subregional analysis of GAD65 mRNA was conducted. Although METH increased GAD65 mRNA levels in both the ventral and dorsal neostriatum, no differences were observed between the two areas. This may be caused by the fact that more distinct changes are observed in the ventromedial subregion or central sectors more lateral to the areas analyzed in our midline sagittal sections (Eisch et al., 1992).

The increase in GABA after METH is likely mediated by D1 receptors within the SNr. These acute increases in SNr extracellular GABA were blocked by local perfusion of the D1 antagonist SCH23390 into the SNr (Fig. 3). Amphetamine increases DA release from dendrites of DAergic neurons in the SNr (Geffen et al., 1976; Heeringa and Abercrombie, 1995). The increase in extracellular DA can then activate D1 receptors present on striatonigral terminals (Porceddu et al., 1986; Altar and Hauser, 1987) to increase extracellular GABA (Aceves et al., 1995; Timmerman

and Abercrombie, 1996; Matuszewich and Yamamoto, 1999). It is uncertain if SNr GABA measured in our study originates from striatonigral or pallidonigral terminals. Because D1 antagonism blocks METH-induced increases in extracellular GABA within the SNr and other findings showing that SNr D1 receptors are located primarily on striatonigral terminals, METH probably activates the striatonigral GABAergic pathway via striatal GAD65 mRNA and a D1-mediated increase in GABA release from striatonigral terminals.

The major projection from the SNr is to the VM (Somogyi et al., 1979; Bevan et al., 1994). GABA tonically inhibits GABAergic neurons in the SNr via GABA-A receptors (Rick and Lacey, 1994). Additionally, intranigral activation of GABA-A receptors located on GABAergic soma within the SNr that project to the motor thalamus decrease thalamic neuron firing (Deniau and Chevalier, 1985). Along these lines, the D1 stimulation increased extracellular GABA concentrations in the SNr and motor activity (Trevitt et al. 2002), the latter presumably mediated through SNr GABAergic neurons that innervate the VM (Faull and Carman, 1968; Beckstead et al., 1979). Therefore, our finding that METH acutely decreases extracellular GABA within the VM (Fig. 5) can be explained by the inhibition of the nigrothalamic pathway resulting from increases in extracellular GABA in the SNr (Fig. 3). GABAergic neurons of the SNr innervate and inhibit VM neurons (Di Chiara et al., 1979), whereas inhibition of SNr activity by intranigral application of GABA increases the activity of a large percentage of thalamocortical neurons (Deniau et la., 1985). Thus, the METH-induced increase in SNr GABA is probably associated with a decrease in extracellular GABA in the VM mediated by a decrease in impulse flow originating from the activation of SNr GABA-A receptors. This interpretation is supported by the finding that perfusion of the GABA-A antagonist BIC into the SNr blocked the METH-induced decrease in extracellular GABA in the VM (Fig. 4). However, a previous study showed that basal extracellular concentrations of GABA in the VM are insensitive to TTX perfusion in the VM, suggesting that basal extracellular GABA measured by microdialysis in the VM is not impulse-derived (Timmerman et al. 1997). The main differences between this study and ours can be explained by the differences between GABA-A receptor-mediated decreases in extracellular GABA originating from the nigrothalamic pathway in the present study and the blockade of sodium channels on all afferents in the VM originating from the globus pallidus, frontal cortex, superior colliculus, and cerebellum (Herkenham, 1979). Regardless, because GABA-A receptor antagonism in the SNr blocked the METH-induced decrease in extracellular GABA in the VM, the decrease in GABA after METH most likely reflects a decrease in GABA-A receptor-mediated and impulse-dependent input to the VM from the SNr.

The decreases in extracellular GABA within the VM after METH (Fig. 5) can alter thalamocortical glutamatergic activity and subsequently, corticostriatal GLU transmission. Because VM glutamatergic neurons innervate the motor cortex (Moran et al., 1982), the METH-induced decrease in GABA in the VM may disinhibit the thalamocortical glutamatergic pathway and increase cortical activity. In fact, METH produces excitotoxicity in the motor cortex, as evidenced by fluorojade immunoreactivity (Eisch et al., 1998) and a long-term decrease in NMDA receptor binding (Eisch et al., 1996). The acute increase in cortical extracellular GLU after METH (Burrows and Yamamoto, 2003) can presumably increase corticostriatal activity and explain the METH-induced increase in extracellular GLU (Nash and Yamamoto, 1992). Moreover, cortical ablation attenuates the METH-induced increases in extracellular striatal GLU (Burrows and Yamamoto, 2003) and suggests that activation of the corticostriatal glutamatergic pathway plays a role in the excitotoxicity to striatal DA terminals.

METH depletes striatal DA content when measured 7 d after drug treatment. A disruption of the METH-induced changes in the striatonigral or nigrothalamic pathways was posited to alter the acute METH-induced increases in extracellular GLU in the striatum and consequently, the long-term depletion of striatal DA content. In fact, D1 antagonism attenuated both the acute METH-induced increase in extracellular GABA in the SNr (Fig. 3) and the long-term depletions of striatal DA tissue content measured 7 d later (Fig. 6). In addition to the blockade of the METH-induced decreases in extracellular GABA in the VM (Fig. 4) by BIC perfusion in the SNr, the acute increase in extracellular GLU (Fig. 5) and the subsequent long-term depletion of striatal DA was also blocked on the side ipsilateral to the local perfusion of BIC (Fig. 7). In contrast, intranigral perfusion of SCH 23390 only attenuated but did not completely block the METH-induced DA depletions in striatum (Fig. 6). One explanation is that BIC more directly and effectively alters the nigrothalamic pathway via convergent inputs from the globus pallidus and striatum onto GABA-A receptors, whereas SCH23390 alters D1-mediated GABA release only from striatonigral terminals to affect nigrothalamic GABAergic transmission.

Although D1 antagonism blocked METH neurotoxicity (Sonsalla et al., 1986; O'Dell et al., 1993), the mechanism of this neuroprotection was undefined. Based on the current findings, the neuroprotection by D1 antagonism can be explained at the level of the SNr to acutely attenuate the METH-induced increase in striatonigral GABAergic transmission (Fig. 3), the subsequent maintenance of inhibitory GABAergic tone in the VM (Fig. 4), and the attenuation of the increase in corticostriatal GLU (Fig. 5). These data are consistent with the findings that NMDA receptor antagonists block METH-induced DA neurotoxicity (Sonsalla et al., 1989; Fuller et al., 1992; O'Dell et al., 1992), presumably mediated by ionotropic NMDA and/or AMPA receptors that trigger a cascade of events including calcium-dependent proteolysis (Si-

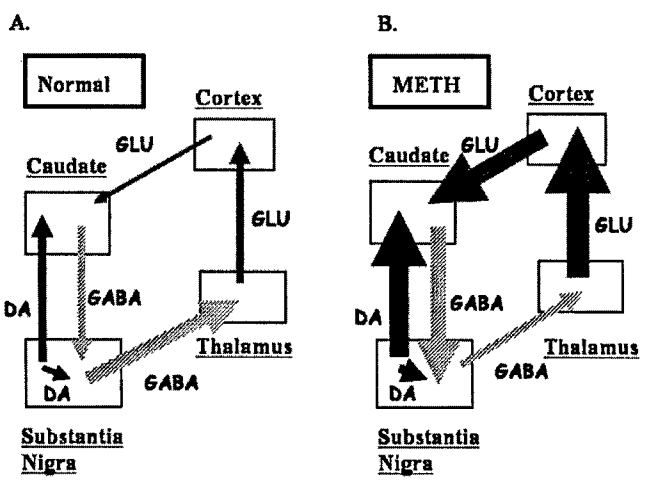

Figure 8. A hypothetical model of polysynaptic effects leading to METH-induced striatal GLU release. Degree of activity is represented by thickness of the arrows. *A*, Normal conditions, under which basal activity of the substantia nigra regulates both DA and GLU release in the striatum. *B*, Effects of METH. DA release in the striatum increases GABAergic release in the SNr, which inhibits nigrothalamic outflow leading to a disinhibition of thalamocortical afferents and subsequent activation of the corticostriatal GLU pathway.

man and Noszek, 1988) and oxidative stress (Fleckenstein et al., 2000; Burrows and Yamamoto, 2003).

In conclusion, long-term striatal DA depletions produced by METH are, in part, caused by activation of the basal ganglia outflow pathway. Figure 8 illustrates that METH (Fig. 8*B*) activates the direct striatonigral GABAergic pathway via increased DA release in the striatum and SNr and activation of D1 receptors in the SNr to inhibit nigrothalamic GABA transmission, a subsequent disinhibition of thalamocortical glutamate release, and an eventual increase in corticostriatal GLU.

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Dopaminergic Regulation of the Rat Subthalamonigral Glutamate Pathway

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List of Abbreviations:

SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulate; STN, subthalamic nucleus; DA, dopamine; GABA, gamma-amino-butyric acid; D_2, dopamine receptor subtype 2; D_1, dopamine receptor subtype 1; SCH23390, (R)-(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride; HPLC, high performance liquid chromatography

The regulation of the glutamatergic projection from the subthalamic nucleus to the substantia nigra was studied with dual-probe microdialysis in the awake behaving rat. Reverse dialysis of the cholinergic receptor agonist carbachol into the subthalamic nucleus transiently increased the extracellular concentrations of glutamate in the substantia nigra and was subsequently followed by an increase in extracellular dopamine. Carbachol-stimulated glutamate release was enhanced and prolonged by perfusion of the selective D_2 dopamine receptor antagonist raclopride into the substantia nigra. In contrast, the D_1 dopamine receptor antagonist SCH-23390 and the $GABA_A$ receptor antagonist bicuculline did not affect carbachol-stimulated glutamate release. It can be concluded that the subthalamonigral pathway is positively regulated at the glutamate soma in the subthalamic nucleus by postsynaptic excitatory cholinergic inputs. Furthermore, these findings suggest that activation of D2 but not D1 or $GABA_A$ receptors via somatodendritically released dopamine in the substantia nigra provides negative feedback control on stimulated glutamate release from subthalamic terminals within the substantia nigra.

Keywords: GABA, dopamine microdialysis, carbchol, substantia nigra, subthalamic nucleus

The subthalamic nucleus (STN) is the only glutamatergic nucleus in the basal ganglia (Smith and Parent, 1988). The activity of subthalamic cells is regulated primarily by an inhibitory projection from the lateral segment of the globus pallidus (Shink et al., 1996) and an excitatory projection from the sensory-motor cortex (Fujimoto and Kita, 1993). Dopaminergic afferents from the substantia nigra pars compacta also have been shown to innervate the STN (Hassani et al., 1997). Furthermore, the STN receives an important excitatory cholinergic projection from the pedunculopontine nucleus (PPN) (Lavoie and Parent, 1994; Mouroux et al., 1995) that is mediated by the cholinergic muscarinic M_3 receptor (Flores et al., 1996).

The STN sends dense excitatory projections to the output nuclei of the basal ganglia including the substantia nigra pars reticulata (SNr) and the medial globus pallidus. The striatum, the substantia nigra pars compacta (SNc), and the frontal cortex also receive subthalamic projections (Kita and Kitai, 1987; Smith et al., 1990). The glutamatergic projection from the STN to the substantia nigra (SN), in this subthalamonigral pathway plays a pivotal role in the firing activity and bursting behavior of the substantia nigra compacta dopamine (DA) cells (Smith and Grace, 1992). Previously, it has been demonstrated that electrical or chemical stimulation of the STN increased somatodendritic release of DA in the SN (Mintz et al., 1986; Rosales et al., 1994). Glutamate, presumably from STN afferents, stimulates SNc DA cells through activation of ionotropic NMDA receptors (Chergui et al., 1994; Overton and Clark, 1991). These receptors are located on the soma and on the long dendritic processes of SNc DA cells, which

enter into and branch within the SNr (Chatha et al., 2000). Glutamate acting on non-NMDA receptors has also been shown to modulate the activity of SNc DA neurons (Christoffersen and Meltzer, 1995; Zhang et al., 1994).

The functional role of somatodendritically released dopamine is less studied than that of dopamine released from axons. DA released in the substantia nigra functions in the feedback regulation of the DA cell activity via an activation of D_2 autoreceptors (Pucak and Grace, 1994) and also in regulating the activity of GABAergic output neurons in the SNr through its actions on D_1 receptors (Abercrombie and DeBoer, 1997; Waszczak, 1990). In addition, somatodendritically released DA in the substantia nigra may also act locally to regulate basal glutamate release from STN axon terminals via D_1 receptors (Rosales et al., 1997). The role of D_2 receptors in the local regulation of subthalamonigral glutamate release in the SN is relatively unknown although it has been shown, in the anesthetized rat, that D_2 receptors may modulate glutamate release in the SNr (Abarca et al., 1995) via their presence as heteroreceptors on asymmetric excitatory terminals on midbrain DA neurons (Pickel et al., 2002). However, no studies to date have directly tested and characterized the simultaneous increase in glutamate and dopamine release in the substantia nigra during stimulation of the STN and the role of substantia nigra D_2 receptors in regulating STN-stimulated glutamate release.

The goal of the present study was to investigate the regulation of the glutamatergic projection from the subthalamic nucleus to the substantia nigra utilizing the technique of dual-probe microdialysis in the awake behaving rat.

Reverse dialysis of the selective D_1 antagonist SCH-23390 or the selective D_2 antagonist raclopride into the SN was used to assess the differential contributions of these two receptor subtypes on glutamate release in the substantia nigra during carbachol-stimulation of the STN. Furthermore, the contribution of GABA on glutamate release induced by STN stimulation was assessed by perfusing the selective $GABA_A$ antagonist bicuculline into the SN during stimulation of the STN. It was posited that the administration of cholinergic receptor agonist, carbachol into the subthalamic nucleus will stimulate glutamate and dopamine release in the substantia nigra. It was further hypothesized that the increase in extracellular dopamine within the substantia nigra negatively modulates the stimulated release of glutamate from the subthalamic terminals via the activation of D_2 receptors.

EXPERIMENTAL PROCEDURES

Materials

Carbamylcholine chloride (carbachol), s-(-)-raclopride, r-(+)-SCH-23390, (+)-bicuculline and Dulbecco's phosphate buffered saline were purchased from Sigma-Aldrich Co., (St. Louis, MO, USA).

Animals

Adult male Sprague-Dawley rats (200-300g; Harlan Sprague Dawley, IN, USA) were used in all experiments. Rats were housed four per cage prior to surgery/dialysis and were maintained on a 12:12 hour light/dark cycle in a temperature and humidity-controlled environment. Food and water were available *ad libitum*. Animal care was in strict accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* which was approved and monitored by the Institutional Animal Care and Use Committee of Boston University.

Microdialysis probe construction

All dialysis probes were of a concentric flow design and were constructed as previously described (Yamamoto and Davy, 1992; Yamamoto and Pehek, 1990).

The length of the dialysis membrane was 1 mm for the STN probes, and 1.5 mm for the SN probes. The dead volume of all probes was calculated so as to synchronize the timing and the initiation of drug perfusion with sample collection.

Surgical probe implantation

Rats were anesthetized with a combination of xylazine (6 mg/kg, i.p.) and ketamine hydrochloride (70 mg/kg, i.p.) and were mounted into a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) for the implantation of the microdialysis probes into the SN and the ipsilateral STN. The skull was exposed, holes were drilled, and probes were slowly lowered to the following co-ordinates aimed at STN: AP -3.8 mm, ML ±2.4 mm as referenced from bregma, DV -7.3 mm as referenced from dura, and at SN (at a 15° lateral angle): AP -5.6 mm, ML ±4.2 mm as referenced from bregma, DV -7.6 mm as referenced from dura (Paxinos and Watson, 1986). The probes were secured to the skull with three stainless steel machine screws and cranioplastic cement. Rats were transferred into the dialysis cages and were allowed to recover over night. Dialysis was conducted on the following morning.

***In vivo* microdialysis**

Microdialysis probes were perfused with a modified Dulbecco's phosphate – buffered saline (138 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 1.2

mM $CaCl_2$, and 5.0 mM *d*-glucose, pH 7.4), for two hours prior to baseline sample collection. The perfusion medium was delivered at a constant rate of 1.5 μl/min using a microinfusion pump (Harvard Apparatus, Holliston, MA, USA). Dialysate samples (20 μl) were collected from the outflow of the probe every 20 minutes. For all experiments, Dulbecco's was perfused alone until stable levels of DA or glutamate levels were achieved in the dialysate during baseline collection. The perfusion medium was then switched to one containing the treatment drug and was reverse dialyzed into the brain for one hour. The perfusion medium was then switched back to Dulbecco's for one hour of post-drug collection. At the end of each experiment, the rats were killed by decapitation and the brains were removed and frozen. Probe placements were verified from frozen 40 μm thick coronal sections.

Measurement of extracellular DA and glutamate

Dialysate aliquots (20 μl) were assayed for DA by high performance liquid chromatography (HPLC) coupled to electrochemical detection. Separation was achieved with a 3 μm C18 column (100 × 2.0 mm; Phenomenex, Torrance, CA, USA) and a mobile phase consisting of 25 mM citric acid, 20 mM sodium acetate, 0.1 mM EDTA, 0.215 mM octyl sodium sulfate, and 5% methanol (pH 3.90). Electrochemical detection was performed with a BAS Model LC-4B electrochemical detector (BAS Instruments, West Lafayette, IN, USA) and a glassy carbon electrode maintained at a potential of 0.6 V. Data were collected

using EZChrom Elite application software (Scientific Software Inc., Pleasanton, CA, USA).

Glutamate was assayed from 20 μl aliquots by HPLC coupled to fluorescence detection following precolumn derivatization with *o*-pthaldialdehyde as described by (Donzanti and Yamamoto, 1988) with slight modifications. A 10μl aliquot of the derivatization reagent was automatically added to the 20μl dialysate sample by an ESA Model 542 autosampler (ESA Inc, Chelmsford, MA, USA), mixed, and allowed to react for exactly 90 seconds before injection onto the column. Separation was achieved with a 3 μm C18 column and a mobile phase consisting of 0.1M sodium phosphate, 0.1 mM EDTA, and 10% methanol (pH 6.65). Fluorescence detection was performed with a Waters 474 Fluorescence Detector (Waters Corp, Milford, MA, USA). The excitation and emission wavelengths were set at 340 and 440 nm respectively. Data were collected using EZChrom Elite application software.

Data Analysis

Glutamate and DA data are presented as a percent of baseline to standardize the points and allow for effective comparisons between the different treatments. Dialysis data is presented as means ± SEM. Statistical significance was determined by a two-way ANOVA with repeated measures coupled with Bonferroni's post hoc test ($p<0.05$).

RESULTS

Effect of carbachol perfusions into the STN on extracellular glutamate concentrations in the SN

The basal concentrations of glutamate in the SN were 1524 ± 324 pg/20 μl. Local perfusions of the cholinergic receptor agonist carbachol (1 mM) into the subthalamic nucleus significantly increased extracellular glutamate in the substantia nigra to 171 ± 37% of basal concentrations ($P < 0.05$) (Fig. 1). The increase in glutamate was transient and was only observed in the first 20 min dialysate sample after the beginning of carbachol perfusions. Glutamate concentrations then decreased toward basal values despite the continued perfusion of carbachol. No effects of carbachol on glutamate release in the SN were observed when it was perfused into regions adjacent to the STN.

Effect of SCH and raclopride perfusions into the SN on carbachol-stimulated subthalamic glutamate release in the SN

Local perfusions of the selective D_2 dopamine receptor antagonist raclopride (100 μM) into the SN enhanced the carbachol-stimulated glutamate release to 197 ± 26% of baseline concentrations ($P < 0.05$). Furthermore, the increase in carbachol-stimulated glutamate was prolonged and remained elevated throughout the perfusion period with raclopride. In contrast, local perfusions of

the selective D_1 dopamine receptor antagonist SCH-23390 (100 μM) into the SN had no effect on carbachol-stimulated subthalamic glutamate release in the SN at any time point (Fig. 2). In the absence of subthalamic stimulation by carbachol, raclopride perfusions did not change extracellular glutamate concentrations in the SN as compared to baseline concentrations (102 ± 8%).

Effect of bicuculline perfusions into the SN on carbachol-stimulated subthalamic glutamate release in the SN

Local perfusions of the selective $GABA_A$ receptor antagonist bicuculline (100 μM) into the SN had no significant effect on carbachol-stimulated glutamate in the SN (126 ± 20% of baseline) (Fig. 3).

Effect of carbachol perfusions into the STN on extracellular dopamine concentrations in the SN

The basal concentrations of dopamine in the SN were 1.4 ± 0.2 pg/20μl. Local perfusion of the cholinergic receptor agonist carbachol (1 mM) into the subthalamic nucleus produced an overall significant increase to 124 ± 7% of basal concentrations of extracellular dopamine in the substantia nigra ($P < 0.05$) (Fig. 4). Dopamine concentrations reached maximum levels forty minutes after

the beginning of carbachol perfusions in the STN. No effects of carbachol on dopamine release in the SN were observed when it was perfused into regions adjacent to the STN.

DISCUSSION

The overall objective of these studies was to investigate the regulation of the glutamatergic projection from the subthalamic nucleus to the substantia nigra. Carbachol perfusions into the STN caused an increase in the extracellular concentrations of glutamate and dopamine in the SN. The increase in extracellular glutamate was transient and returned toward basal values despite the continued perfusions of the STN with carbachol. Carbachol-stimulated glutamate release was enhanced and prolonged by perfusion of the selective D_2 dopamine receptor antagonist raclopride into the SN. In contrast, the D_1 dopamine receptor antagonist SCH-23390 and the $GABA_A$ receptor antagonist bicuculline did not affect carbachol-stimulated glutamate release.

Consistent with previous studies using carbachol microinjections into the STN (Flores et al., 1996; Rosales et al., 1997), reverse dialysis of carbachol into the STN increased extracellular glutamate concentrations in the SN. These effects of carbachol are most likely mediated via the activation of muscarinic M_3 cholinergic receptors present in the STN (Flores et al., 1996) and not nicotinic cholinergic receptors since the latter do not contribute significantly to the activity of subthalamic neurons (Feger et al., 1979).

Although reverse dialysis of carbachol in the STN increased the extracellular concentrations of glutamate in the SN, the increase was transient and returned to basal values despite the continued perfusion of carbachol. One possible explanation is that cholinergic receptor desensitization in the STN may

have contributed to the lack of a sustained increase in extracellular glutamate within the SN. However, this possibility is unlikely since local pressure application of high concentrations (10 mM) of carbachol did not reduce the firing of subthalamic neurons (Falkenburger et al., 2001). Another possibility that was considered and addressed in the current study is that the subthalamonigral glutamatergic projection is tightly regulated by an inhibitory dopaminergic feedback mechanism initiated within the substantia nigra at the glutamatergic terminals.

Previous studies have shown that somatodendritically released DA within the SN has been associated with the regulation of SNc DA and SNr GABA cell activity (Abercrombie and DeBoer, 1997; Waszczak, 1990). Some studies have suggested that dopamine can regulate basal glutamate release from STN axon terminals (Abarca et al., 1995; Rosales et al., 1997). The current study extended these findings to show that antagonism of D_2 receptors within the SN enhances and prolongs the increase in glutamate produced by carbachol stimulation of the STN. Therefore, the increase in dopamine observed within the SN during carbachol stimulation of the STN (Fig. 4) may limit the increase in glutamate via activation of D_2 receptors localized presumably on asymmetric excitatory terminals (Pickel et al., 2002).

In contrast, perfusion of the D_1 receptor antagonist SCH-23390 into the SN did not enhance or prolong the transient increase in extracellular glutamate produced by carbachol. These findings do not suggest a role for D_1 receptors in the regulation of glutamate release from STN terminals as proposed previously

(Rosales et al., 1997). The major difference between the current findings and those of Rosales et al. (1997) is that they evaluated the role of D_1 receptors on basal glutamate concentrations whereas we examined whether D_1 receptors play a role on stimulated glutamate release. Therefore, D_1 receptors may play a role in the regulation of basal glutamate concentrations but not under stimulated conditions when the STN is activated.

Collectively these results provide the first evidence to suggest that somatodendritically released dopamine in the SN through an activation of D_2 dopamine heteroreceptors provides a negative feedback control on stimulated glutamate release from STN terminals within the SN. This dopaminergic regulation of glutamate release however, appears to be dependent on subthalamic cell activity since D_2 dopamine receptor blockade did not affect the basal concentrations of extracellular glutamate in the SN in the absence of STN stimulation.

The observed changes in extracellular glutamate in the SN were designed to be selectively reflective of stimulation of the STN. Although additional glutamatergic projections to the SN that originate from the frontal cortex (Carter, 1982) and the pedunculopontine nucleus (Tokuno et al., 1988) may also contribute to the basal concentrations of extracellular glutamate in the SN, it is unknown if these or other glutamatergic projections are under the same dopaminergic control as those originating from the STN. Although adjacent areas to the STN may have unknown glutamatergic projections to the SN or may affect STN activity, our findings illustrate that when regions adjacent to the STN

were perfused with carbachol (Fig. 1), no changes in glutamate concentrations were observed in the SN. Therefore, the increases in glutamate within the SN observed during carbachol perfusions most likely originate from the STN.

Another possibility that was considered is that the regulation of subthalamonigral glutamate transmission can be mediated by the inhibitory neurotransmitter GABA through the direct activation of GABA heteroreceptors on the STN axon terminals or through the modulation of dopamine concentrations in the SN. GABAergic input into the SN originates from the striatum and pallidum (Bolam and Smith, 1990; Smith and Bolam, 1990) and from intrinsic GABAergic interneurons (Grofova et al., 1982). GABA is critically involved in the regulation of DA cell activity by tonically suppressing the excitatory inputs (Kitai et al., 1999). This effect is mediated primarily via $GABA_A$ receptor activation localized on DA cell bodies (Paladini et al., 1999; Tepper et al., 1995). In the present study, perfusion of the $GABA_A$ receptor antagonist bicuculline into the SN during carbachol stimulation of the STN had no effect on glutamate release produced by STN stimulation. This indicates that GABA does not appear to directly regulate glutamate release from STN axon terminals and further highlights the importance of dopamine in regulating glutamate release in the SN during STN stimulation.

The increase in extracellular dopamine within the SN during carbachol stimulation of the STN is consistent with previous findings. Glutamatergic fibers from the STN directly synapse onto the soma and dendrites of DA neurons (Smith et al., 1996). In addition, glutamate is known to increase the activity of SNc DA cells and cause the release of somatodendritic DA mainly by activating

ionotropic NMDA receptors (Chergui et al., 1994; Overton and Clark, 1991) present on the somatodendritic processes of SNc DA cells. Furthermore, stimulation of the STN increases extracelullar dopamine within the SN (Mintz et al., 1986) via the activation of NMDA receptors (Rosales et al., 1994). Moreover, the observed sequence of events in the current study demonstrating that the maximal increase in DA concentrations occurred forty minutes after the beginning of the carbachol STN perfusions and followed the rise in glutamate suggests that the increase in dopamine was a result of the carbachol-induced STN stimulation of glutamate release in the SN.

The current findings have implications for the pathophysiology and pharmacotherapy of Parkinson's disease (PD). It has been hypothesized that the progressive loss of SNc DA cells in PD states results from an increased glutamate transmission in the SN and a subsequent excitotoxic cell death (Rodriguez et al., 1998). Moreover, lesions or high frequency stimulation of the STN reduce most of the motor impairments associated with PD (Benazzouz and Hallett, 2000; Parkin et al., 2001), presumably by reducing the activity of the subthalamonigral pathway. Based on these past findings and the current study, modulation of the excitatory cholinergic stimulation of the subthalamonigral glutamatergic pathway at the level of the STN may be therapeutically beneficial. Perhaps more important however, is that modulation of the glutamate release by the D_2 receptor within the SN occurs only under conditions of STN stimulation and suggests that the subthalamonigral glutamatergic pathway can be tightly regulated primarily at the axon terminal region by presynaptic inhibitory D_2

heteroreceptors. Thus, these findings further support the use of D_2 agonists that target not only the striatum but also target the SN for the modulation of aberrant or augmented subthalamonigral glutamate release in relation to the symptomatic treatment of PD and possibly for the prevention of the progressive excitotoxic degeneration of dopamine cell bodies in the substantia nigra.

Acknowledgements: This work was supported by DA07606, DAMD 17-99-1-9479 and a gift from Hitachi America Inc.

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FIGURE LEGENDS

Fig. 1. Effect of carbachol perfusions into the subthalamic nucleus on extracellular glutamate concentrations in the SN. Local carbachol (1mM) perfusions into the STN are represented by the closed circles (n=8). Controls (open circles) are carbachol perfusions into regions adjacent to the STN (n=7). Glutamate data are presented are percent of baseline. All data are mean ± SEM. * P< 0.05.

Fig. 2. Effect of SCH and raclopride perfusions into the SN on carbachol-stimulated subthalamic glutamate release in the SN. Local perfusions of raclopride (100 μM) into the SN are represented by the open circles (n=6). Local perfusions of SCH -23390 (100 μM) into the SN are represented by the closed triangles (n=5). Control values for this figure (closed circles) are aCSF perfusions into the SN and are reproduced from figure 1 (n=8). Glutamate data are presented are percent of baseline. All data are mean ± SEM. * P< 0.05.

Fig. 3. Effect of bicuculline perfusions into the SN on carbachol-stimulated subthalamic glutamate release in the SN. Local perfusions of bicuculline (100 μM) into the SN are represented by the open circles (n=5). Control values for this figure (closed circles) are aCSF perfusions into the SN and are reproduced

from figure 1 (n=8). Glutamate data are presented are percent of baseline. All data are mean ± SEM. * P< 0.05.

Fig. 4. Effect of carbachol perfusions into the STN on extracellular dopamine concentrations in the SN. Local perfusions of carbachol into the STN are represented by the closed circles (n=7). Controls (open circles) are carbachol perfusions into regions adjacent to the STN (n=5). Dopamine data are presented are percent of baseline. All data are mean ± SEM. * P< 0.05.

Figure 1

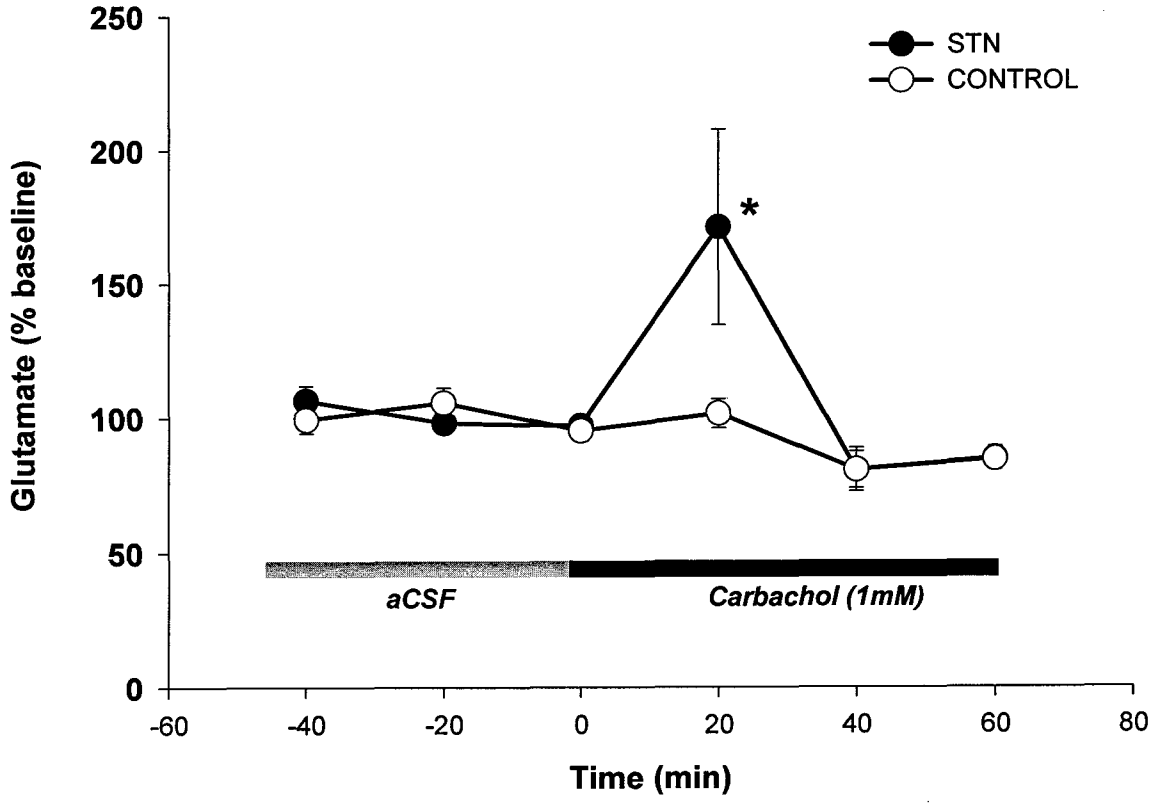

250
200
150
100
50
0
Glutamate (% baseline)
STN
CONTROL
*
aCSF
Carbachol (1mM)
-60
-40
-20
0
20
40
60
80
Time (min)

Figure 2

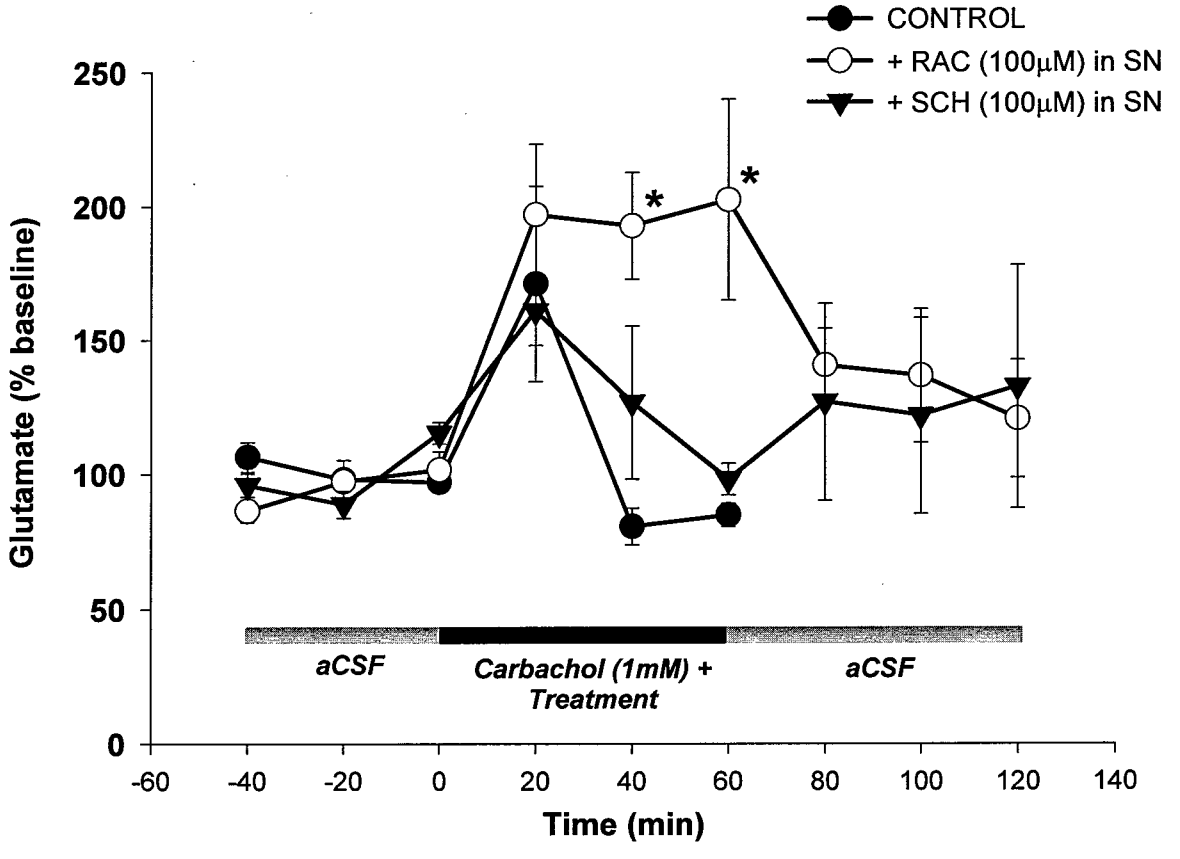

CONTROL
+ RAC (100μM) in SN
+ SCH (100μM) in SN
Glutamate (% baseline)
250
200
150
100
50
0
-60
-40
-20
0
20
40
60
80
100
120
140
Time (min)
*
*
aCSF
Carbachol (1mM) +
Treatment
aCSF

Figure 3

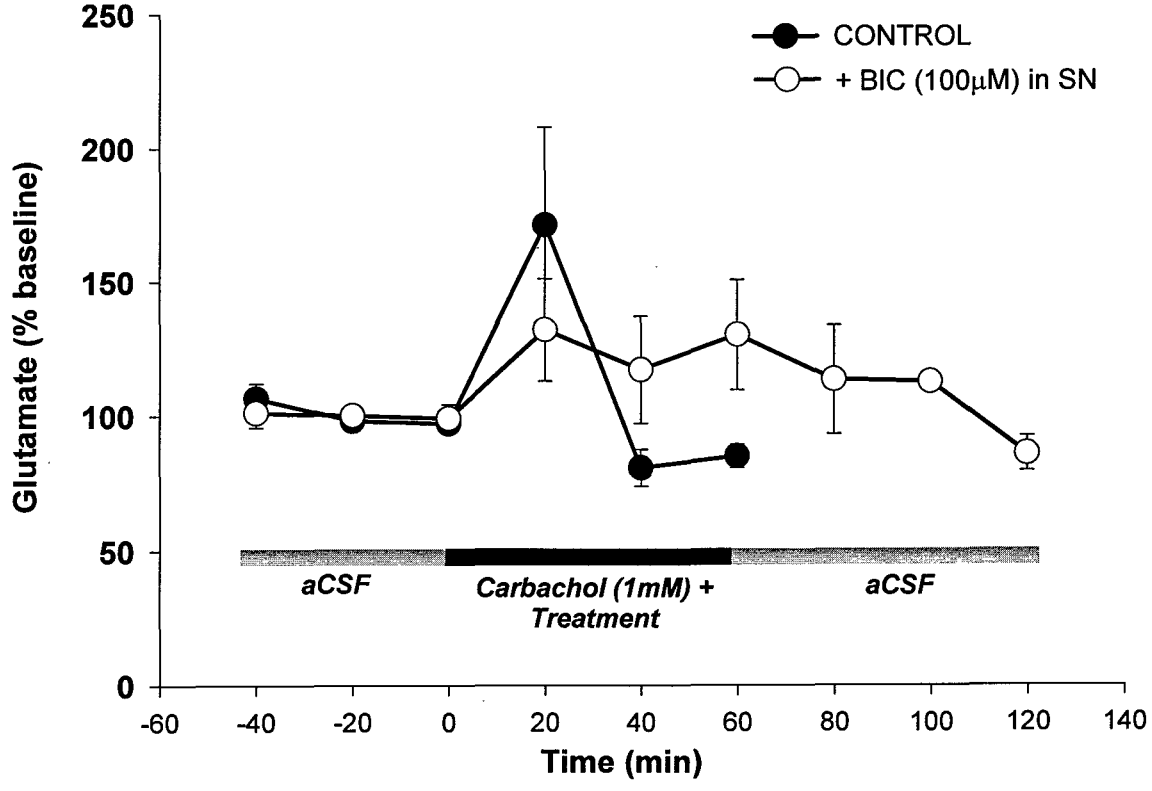
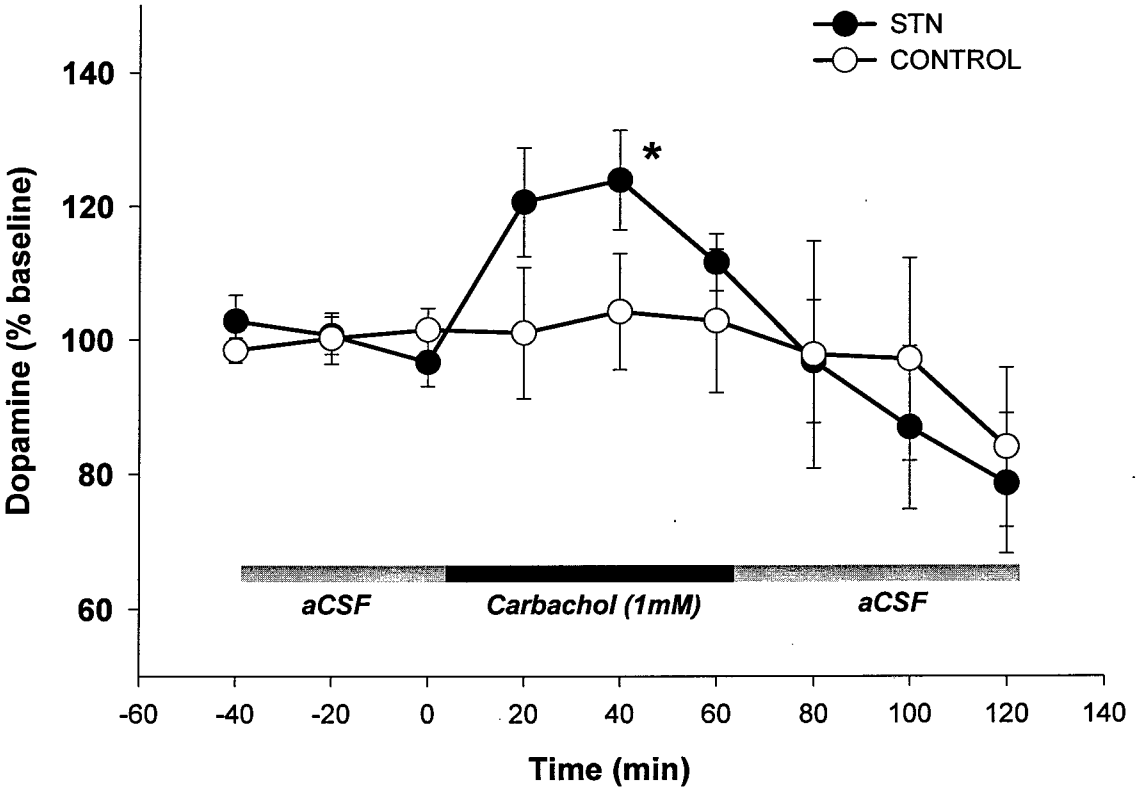

CONTROL
+ BIC (100μM) in SN
Glutamate (% baseline)
250
200
150
100
50
0
-60
-40
-20
0
20
40
60
80
100
120
140
Time (min)
aCSF
Carbachol (1mM) + Treatment
aCSF

Figure 4


STN
CONTROL
*
140
120
100
80
60
Dopamine (% baseline)
aCSF
Carbachol (1mM)
aCSF
-60
-40
-20
0
20
40
60
80
100
120
140
Time (min)

METHAMPHETAMINE-INDUCED INHIBITION OF MITOCHONDRIAL COMPLEX II: ROLES OF GLUTAMATE AND PEROXYNITRITE

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Nonstandard abbreviations: Methamphetamine (METH); Electron Transport Chain (ETC); 3-nitropropionic acid (3NP), 5, 10, 15, 20-tetrakis (2,4,6-trimethyl-3,5-sulfonatophenyl) porphinato iron III, (Fe-TPPS).

Abstract:

High-Dose methamphetamine (METH) is associated with long-term deficits in dopaminergic systems. Although the mechanism(s) which contribute to these deficits are not known, glutamate and peroxynitrite are likely to play a role. These factors are hypothesized to inhibit mitochondrial function, increasing the free radical burden and decreasing neuronal energy supplies. Previous studies suggest a role for the mitochondrial electron transport chain (ETC) in mediating toxicity of METH. The purpose of the present studies was to determine if METH administration selectively inhibits complex II of the ETC in rats. High-dose METH administration (10 mg/kg every 2 h × 4) rapidly (within 1 h) decreased complex II (succinate dehydrogenase) activity by ~20-30%. In addition, decreased activity of complex II-III, but not complex I-III, of the mitochondrial ETC was also observed 24 h after METH. This inhibition was not due to direct inhibition by METH or METH-induced hyperthermia and was specific to striatal brain regions. METH-induced decreases in complex II-III were prevented by MK-801 and the peroxynitrite scavenger, Fe-TPPS. These findings provide the first evidence that METH administration, via glutamate receptor activation and peroxynitrite formation, selectively alters a specific site of the ETC.

Running title: Methamphetamine Inhibits Complex II

Keywords: Electron Transport Chain; Free radicals; Peroxynitrite; Mitochondria; Neurodegeneration; Succinate Dehydrogenase.

High-dose methamphetamine (METH) administration results in long-term deficits in monoaminergic systems (Hotchkiss et al., 1979; Ricaurte et al., 1982; Ricaurte et al., 1984; Wagner et al., 1980; Axt et al., 1991; Villemagne et al., 1998; McCann et al., 1998 2; Frey et al., 1997). Although these deficits have been described, the mechanism(s) involved in mediating these changes remain largely unknown. However, numerous factors have been implicated including glutamate (Nash and Yamamoto, 1992) and reactive nitrogen species (i.e. peroxynitrite) (Imam and Ali, 2001) which have been hypothesized to converge at a single target, mitochondria, to produce damage to neuronal elements (for review see Brown and Yamamoto, 2003). Therefore, mitochondrial inhibition may represent the initial catalytic site which mediates these long-term deficits in markers of striatal dopaminergic terminals.

Mitochondria regulate ATP synthesis via the mitochondria electron transport chain (ETC). The ETC is a series of four complexes (I-IV) which pass free electrons via oxidative phosphorylation to form ATP. The products of this oxidative phosphorylation include water and numerous free radicals such as hydroxyl and superoxide radicals. In fact, it has been speculated that mitochondria represent the largest source of endogenous free radicals (Chance, 1979). Furthermore, inhibition of normal ETC function, or even specific complexes within the ETC, can increase free radical production. For example, inhibition of complex II with 3-nitropropionic acid (3NP) increases free radical production within neurons of the striatum (Kim et al., 2002).

Recent studies provide suggestive evidence that high-dose METH administration alters complex II within the ETC, an effect that may contribute to the long-term deficits associated with METH. For example, direct infusion of METH into the striatum does not result in long-term depletions of dopamine however, direct infusion of METH with the complex II inhibitor, malonate, results in long-term depletions of striatal dopamine content that are greater than that seen with malonate alone (Nixdorf et al., 2001). In addition, the combination of METH with a low dose of 3NP markedly increased the frequency of striatal lesion formation compared to 3NP administration alone (Reynolds et al., 1998). Furthermore, parallel lines of evidence suggest that

the effects of inhibition of complex II and METH administration are similar. Both complex II inhibition and METH result in loss of markers for dopamine terminals in the striatum without affecting dopamine cell bodies in the substantia nigra (Zeevalk et al. 1997; Blum et al., 2004; Ricaurte et al., 1982). Thus, it is possible that METH produces damage to dopaminergic terminals in the striatum through the inhibition of complex II.

The present studies were conducted to directly test the hypothesis that high-dose METH administration rapidly inhibits complex II enzyme function. Results demonstrated that METH decreased complex II activity in a manner dependent on glutamate-receptor activation and peroxynitrite formation. These findings provide the first direct evidence that METH administration selectively alters a specific site of the ETC and implicates mitochondrial inhibition as an early event in the toxicities associated with high-dose METH administration.

METHODS:

Animals:

Male Sprague-Dawley rats (250-300 g; Charles River Laboratories) were maintained on a 12 h light/dark cycle in a temperature- and humidity-controlled environment. Rats were initially housed two to four per cage in plastic cages with food and water available ad libitum. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Boston University Medical School institutional animal care and use committee.

Treatments:

High-dose METH was administered in a series of four single injections (1 injection every two h) of 10mg/kg s.c. ll rats were killed by decapitation 1 or 24 h following the last drug administration. Vehicle (saline) administrations were given in a corresponding manner at dose of 1ml/kg, s.c.. MK-801 (1 mg/kg, i.p.) was administered 1 h and 5 h after the last METH administration. These time points and doses were selected based on the half-life of MK-801 (Vezzani et al., 1989) and the delayed rise in extracellular glutamate after high-dose METH administration (Nash and Yamamoto, 1992). 5, 10, 15, 20-tetrakis (2,4,6-trimethyl-3,5-sulfonatophenyl) porphinato iron III, Fe-TPPS, a specific peroxynitrite decomposition catalyst (Jensen and Riley, 2002) was administered (10 mg/kg, i.p.) 12 hrs prior to the first and 5 min prior to the third METH administration. These doses were selected based upon previously published data demonstrating the ability of a similar dosing regimen to prevent METH-induced nitrotyrosine formation and dopaminergic deficits (Imam et al., 2001b). In experiments where METH-induced hyperthermia was prevented, one cage containing METH-treated animals was placed on ice during the entire dosing regimen (normothermic and METH-treated). Rectal temperatures were monitored throughout the treatment period.

Preparation of mitochondria:

Striatal and hippocampal tissues were removed and rapidly frozen on dry-ice. No differences in baseline enzyme rates were seen between fresh and previously frozen tissue (data not shown). Once all samples were collected, tissues were homogenized by hand using a glass homogenizer and with Teflon pestle in homogenization buffer (final concentrations: 1% cholate and 30 mM potassium phosphate pH 7.5) at a concentration of 50 mg/ml (original tissue wet weight) and incubated on ice for 5 min. The homogenate was centrifuged at 4°C (14,000 x g 5 min) to remove membranous fragments. The supernatant, which represents a crude mitochondrial preparation from various cell types, was used for all subsequent assays.

Complex I-III/II-III Assay

Activity of mitochondria complexes I-III and II-III were assessed as described previously (Sottocasa et al., 1967) with the following modifications. An aliquot (50μL) of the homogenate was added to the appropriate incubation buffer. The buffer was prepared fresh for each assay (Final concentrations: EDTA 100μM, BSA 0.2%, and potassium phosphate 50mM pH 7.5 @ 37°C, cytochrome C 150μM, and asolectin 150μg/ml). After preincubation (37°C 3min), substrate was added for the measurement of complex I-III, (NADH, 2 mM final concentration) or complex II-III (succinate, 40 mM final concentration). All assays contained potassium cyanide to inhibit complex IV (1.5mM final concentration). Upon addition of substrate, samples were placed in a Versamax plate reader (Molecular Probes) and reduction of cytochrome C was measured at 550 nm for 10 min at 37°C. Because of the poor solubility of ETC inhibitors in aqueous solutions, enzymes rates were calculated by subtraction of activity in the absence of substrate (i.e. succinate or NADH) from substrate dependent activity. No differences were noted in enzyme rates in the presence of inhibitor or in the absence of substrate (data not shown). All

samples were normalized to protein concentrations as determined using a commercially available Bradford protein assay (Bio-Rad). All samples were run in triplicate and average rates calculated.

In vitro peroxynitrite experiments were conducted on mitochondria isolated as described above. The concentration of peroxynitrite (Upstate, Charlottesville, VA) was determined just prior to each treatment. Mitochondria were incubated with increasing concentrations of peroxynitrite for 3 min prior to addition of substrate. Activity was assessed as described above.

Spectrophotometric Succinate Dehydrogenase Assay

Activity of complex II (succinate dehydrogenase) was assessed as described previously (Hoppel and Cooper 1968). Briefly ~100 μg protein was incubated in Tris buffer (final concentration: 100mM-0.1% Tris-BSA pH 7.8; 0.5mM phenazine sulphate, ~ 1mM DCPIP) and incubated at 37^{0}C for 5 min. The reaction was initiated by the addition of succinate (40 mM final). Enzyme activity was monitored spectrophotometrically by the reduction of DCPIP/PES at 600 nm. Enzyme rates were calculated from the reciprocal of the slope and normalize to protein as described above.

Histochemical Succinate Dehydrogenase Assay

Histochemistry was performed according to the methods described by Brouillet et al. (1998). One hour following METH treatment, rats were killed by decapitation, brains removed and rapidly frozen on dry ice. 40 μM sections were collected and mounted on poly-L-lysine coated slides. To assess succinate dehydrogenase activity sections were air-dried and incubated for 15 min in 37°C PBS (0.1M PBS in 0.9% NaCl) followed by a 30-min incubation in 0.3 mM nitroblue tetrazolium (NBT); 0.05 M sodium succinate and 0.05 M phosphate buffer, pH 7.6. For the 3-NP experiments, 1μM or 10 mM 3-NP was added to the incubation buffer. All slides were rinsed for 5 min in ice-cold PBS followed by a 5 min rinse in ddH_2O, then allowed to air dry.

Dried slides were rinsed in Xylene and coverslipped with Permount. Optical density for each section was determined using Image J software.

Statistics:

Results were compared using a Students' t-test or ANOVA followed by a Student-Newman-Kuels post-hoc analysis or a two-way ANOVA as indicated. Significance was set at $p \leq 0.05$.

RESULTS:

To determine if high-dose METH administration alters mitochondrial ETC activity, rats were treated with high-dose METH and mitochondrial activity was determined 1 h after the last drug administration. Complexes II-III, but not I-III, were rapidly (within 1 h) inhibited by high-dose METH administration (figure 1). To examine the role of METH-induced hyperthermia in the decrease in complex II-III activity, a third group was included in this same experiment, whereby METH-induced hyperthermia was prevented (see methods). Average rectal temperatures for each treated group were as follows: Sal 37.2±0.3°C, METH hyperthermic 39.8±0.6°C ($p<0.001$ verse SAL), METH normothermic 37.7±0.7°C ($p<0.001$ verse METH alone). Prevention of METH-induced hyperthermia did not reverse the METH-induced decrease in complex II-III activity (figure 1). The inhibition of complex II-III activity is specific for striatal, but not hippocampal, brain regions (figure 2) and is unlikely due to residual METH in the mitochondrial preparation. This latter statement is based on the finding that direct application of METH to the homogenate in concentrations up to ~1 mM did not affect complex II-III activity (figure 3).

Because decreases were observed in complex II-III but not complex I-III, it was hypothesized that the METH-induced inhibition was specific for complex II. To test this hypothesis, complex II activity (i.e. succinate dehydrogenase) was examined independent of complex I or III. Use of specific complex inhibitors confirmed the selectivity of this assay for complex II (Table 1). Data presented in figure 4 demonstrate that METH inhibits the activity of complex II. In an attempt to localize the effect on METH on complex II within the striatum, rats were treated with high-dose METH and histochemical analysis for striatal succinate dehydrogenase activity was performed. Results demonstrate no significant decrease in succinate dehydrogenase activity in the striatum following METH using histochemical methods (Table 2). In vitro incubation of striatal slices with increasing concentrations of 3NP show that 1μM 3NP produced a 16 % inhibition of succinate dehydrogenase activity ($p<0.001$), while the 10mM

concentration produced a 46 % inhibition ($p<0.001$) using the densitometric histochemical method. These findings are in contrast to data presented in Table 1 (1uM 3NP = 75% inhibition of succinate dehydrogenase activity) and suggest a greater sensitivity of the biochemical vs. histochemical methods for detecting changes in succinate dehydrogenase activity.

Previous studies examining the effect of METH on mitochondrial function suggest that infusion of mitochondrial substrates hours after the high-dose METH treatment afforded protection against METH-induced dopaminergic deficits (Stephans et al., 1998). In addition, high-dose METH caused a delayed raise in extracellular glutamate (Nash and Yamamoto, 1992) which can persist for at least 12 hours after METH administration (unpublished observation). Previous studies examining the effect of glutamate receptor stimulation demonstrate a specific inhibition of complex II enzyme activity (Dabbeni-Sala et al., 2001). These data suggest that delayed increases in striatal glutamate following METH may contribute to the selective decrease in complex II activity. To assess the role of long-term complex II inhibition and delayed glutamate elevation, complex II-III activity was assessed 24 h following high-dose METH administration in the presence and absence of the glutamate receptor antagonist MK-801. This time point was selected to represent the latest time point after METH administration at which dopamine terminal degradation has not occurred. Results demonstrate that complex II-III activity (figure 6) was decreased 24 h following high-dose METH administration as compared to saline controls. Late MK-801 treatment restored complex II-III activity to control levels (figure 5). No difference was seen between saline and late MK-801 treatment in the absence of METH (figure 5).

NMDA receptor activation has been linked to activation of nitric oxide synthase (NOS) (Ayata et al., 1997), which can increase reactive nitrogen species like peroxynitrite. Since peroxynitrite has been implicated in METH-induced deficits, we examined the direct effects of peroxynitrite on complex I-III and II-III activity. Results presented in figure 6 demonstrate the direct incubation with peroxynitrite dose-dependently inhibited complex II-III with little effect on

complex I-III activity at these ONOO- concentrations tested. To determine the role of peroxynitrite in the METH-induced decrease in complex II-III, the specific peroxynitrite scavenger Fe-TPPS was administered as described in the Methods section. Treatment with Fe-TPPS restored complex II-III activity to control levels (figure 7). No difference was seen between saline and Fe-TPPS treatment in the absence of METH.

DISCUSSION:

High-dose METH administration rapidly and selectively inhibited the activity of complex II in the mitochondrial ETC (figure 1). This inhibition of complex II activity was specific for striatal, but not hippocampal brain regions (figure 2) and is unrelated to METH-induced hyperthermia (figure 1) or residual METH in the mitochondrial preparation (figure 3).

The early decreases (i.e. within 1h) in complex II activity observed represented a decrease of ~25-30% at 1 h. This change may not reflect the actual magnitude of the METH-induced decrease since the mitochondrial preparation utilized in the present studies represents numerous cell types within the striatum. Attempts to localize the METH-induced decrease in succinate dehydrogenase using histochemical techniques showed no effect of METH treatment (Table 2). However, histochemical methods for detecting changes in succinate dehydrogenase appeared less sensitive than biochemical methods since the same concentration of 3NP produced changes in succinate dehydrogenase activity of much smaller magnitude than those observed using the biochemical analysis. Given this fact, it is not surprising that the ~25-30% decrement in complex II was not detected via histochemical techniques. There are no current methods for isolating mitochondria from a specific cellular population. Therefore, the exact location of the affected mitochondria and the exact decrease in complex II are difficult to ascertain. However, numerous studies support a role of mitochondrial inhibition in mediating the long-term dopaminergic deficits induced by METH. Agents which support mitochondrial respiration protect against METH-induced dopaminergic deficits (Stephans et al., 1998) such that the infusion of decylubiquinone, which serves as an electron donor downstream of complex II, completely protected against METH-induced deficits only when infused after the METH administrations. Furthermore, intracranial infusion of METH only caused long-term deficits in dopaminergic systems when co-administered with the specific complex II inhibitor malonate (Nixdorf et al., 2001). These results suggest that a delayed and prolonged inhibition of complex II contributes to the METH-induced dopaminergic deficits. Moreover, complex II inhibition by

either malonate or 3NP infusion selectively damages striatal dopaminergic terminals and not dopamine cell bodies in the substantia nigra suggesting an increased vulnerability of dopamine terminals to complex II inhibition (Zeevalk et al., 1997; Blum et al., 2004). This is consistent with the apparent selective nature of METH-induced deficits at dopamine terminals (Ricaurte et al., 1982). The present study extends these findings by demonstrating an early but prolonged (up to 24 h) and selective inhibition of complex II after METH administration. These changes at 1 and 24 hr after METH precede any evidence of dopaminergic terminal degeneration and suggest that complex II inhibition represents a precursor event of METH toxicity that is not simply a consequence of dopamine terminal loss and implicates the METH-induced inhibition of complex II in the long-term deficits induced by high-dose METH administration.

Several experiments were conducted to elucidate the mechanism mediating the decrease in complex II activity. The observed effects are not due to a direct effect of METH, since incubation of mitochondria with concentrations of METH that are typically achieved in suspensions of striatal tissue (~48nM) after a systemic injection regimen (Riddle et al., 2002) had no effect on complex II-III or I-III activity. Only millimolar concentrations of METH produced a non-selective inhibition of complexes I-III (data not shown) and II-III (figure 3). In addition, the decrease in complex II-III was not associated with METH-induced hyperthermia since prevention of hyperthermia did not reverse the decrease in complex II-III activity (figure 1). Previous studies showed that prevention of hyperthermia is neuroprotective against METH-induced dopaminergic deficits (Albers and Sonsalla, 1995). However, it was concluded in these studies that hyperthermia contributes to, but is not solely responsible for, METH-induced deficits (Albers and Sonsalla, 1995). Thus, the decrease in complex II-III may mediate a hyperthermia-independent component of METH toxicity to dopaminergic elements. Alternatively, hyperthermia may enhance the toxic effects of mitochondrial inhibition but does not contribute directly to mitochondrial inhibition.

Although the exact mechanism of this decrease in complex II remains to be determined, METH-induced increases in striatal glutamate appear to contribute. Specifically, administration of an NMDA receptor antagonist MK-801, even injected *after* the administration of METH, prevented METH-induced decreases in activity of complex II-III (figure 5). Dabbeni-Sala et al. (2001) demonstrated that incubation with a glutamate receptor agonist causes a selective loss of complex II activity. These effects were prevented by a glutamate receptor antagonist and antioxidant treatment (i.e. melatonin or GSH). METH produces a delayed increase in striatal glutamate (Nash et al., 1992), which can, in turn, activate glutamatergic ionotropic receptors and damage dopamine terminals in the striatum. This increased stimulation of glutamate receptors may initiate a feed-forward mechanism that occurs predominately *after* the METH administration regimen and results in inhibition of complex II. The finding that administration of MK-801 *after* METH can prevent the decrease in complex II activity is consistent with the delayed rise in glutamate (Nash and Yamamoto, 1992) and suggests that late occurring excitotoxic events to complex II can still be blocked pharmacologically. These data may have significant implications for the treatment of METH overdose.

Glutamate-mediated receptor activation has been linked with NOS activation, protein nitration and peroxynitrite formation in the striatum (Ayata et al., 1997). Numerous studies have implicated NOS and peroxynitrite as key players in protein nitration and METH-induced deficits (Imam et al., 2001b). Results presented in figure 5 demonstrate that complex II-III activity showed a greater sensitivity to inactivation by ONOO- when compared to complex I-III activity. A greater sensitivity of complex II to ONOO- has been shown previously by others. For example, Murray et al. (2003) demonstrated that complex I and II were equally inhibited by incubation with 800 μM ONOO but complex II showed a greater inhibition at higher concentrations (1.6mM ONOO-). These results parallel the finding in the present study (Figure 6). In addition, results from Cassina and Radi (1996) demonstrated a preferential inhibition of complex II when compared to complex I by peroxynitrite. It should be noted that previous studies have

demonstrated inhibition of complex I by reactivity nitrogen species (see Murray et al. (2003)). At the doses on ONOO- tested a trend, albeit not significant, decrease in complex I-III activity was noted. Alternatively, METH-induced glutamate release could also lead to inhibition of complex II through the increased intracellular Ca^{2+} concentrations (Kushnareva et al., 2005; Ward et al., 2005) and generation of reactive oxygen species (Kahlert et al., 2005). Furthermore, Ayata et al. (1997) showed that NMDA receptor activation in nNOS knockout mice could still produce damage to the striatum, despite the absence of protein nitration. Although the size of the lesions was reduced by 50% when compared to the wild-type animals that exhibited extensive protein nitration, these results suggest that glutamate could also lead to neuronal toxicity that is independent of NOS activation. In some experiments presented in the current study a non-significant trend for decreases in complex I-III was noted. Therefore the potential exists for some inhibition of complex I-III activity but this inhibition appears less than that of complex II.

The link between peroxynitrite formation and METH-induced inhibition of complex II-III activity and METH was further supported by administration of the peroxynitrite scavenger Fe-TPPS. When administered 12 hr before the first and 5 min prior to the third METH administration, this agent reversed the METH-induced decrease in complex II-III activity (Figure 6). Interestingly, Fe-TPPS has also been shown to prevent the dopaminergic deficits induced by high-dose METH administration (Imam et al., 2001b), further supporting the link between METH, complex II inhibition, peroxynitrite, and long-term dopaminergic deficits.

Although numerous links have been made between METH, peroxynitrite and dopaminergic deficits, few studies have examined the role of peroxynitrite in METH-induced serotonergic toxicity. Studies by Abekawa et al. (1996) have demonstrated that inhibition of nitric oxide, the precursor to peroxynitrite, attenuated dopamine but not serotonin toxicity, following high-dose METH administration. Similarly Itzhak et al., (2003) demonstrated that removal of the NOS gene is not protective against serotonergic deficits induced by administration of the amphetamine analog, fenfluramine. It was concluded in this study that peroxynitrite does not

mediate fenfluramine-induced serotonergic neurotoxicity but may be a specific mediator of amphetamine-induced dopaminergic neurotoxicity (Itzhak et al., 2003). Although speculative, this selective effect of peroxynitrite-mediated, dopaminergic toxicity produced by amphetamine may explain the lack of an effect of METH on complex II-III in hippocampal brain regions seen in the present studies.

Unlike other ETC complexes, little is known about the regulation of complex II by pharmacological agents. Although agents such as malonate directly inhibit complex II activity, the present study demonstrates that other pharmacological agents such as METH can indirectly, but selectively inhibit this complex through glutamate-, peroxynitrite-mediated mechanisms. This rapid and persistent loss of complex II activity produced by METH may have long-term consequences on neuronal function. These studies provide the first direct evidence that high-dose METH rapidly decrease complex II activity via glutamate-receptor, peroxynitrite-mediated mechanisms. This inhibition may represent the initial catalytic event that occurs predominately after and not during METH administration and contributes to METH-induced toxicities and perhaps other neurodegenerative disorders.

Acknowledgements:
This research was supported by PHS grants DA 07606, DAMD17-99-19479, and a gift from Hitachi America, Inc.

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Effect of ETC Inhibitors on Complex II Activity

	Final Concentration	%	STDEV
Control	-	100.00	(14.11)
DMSO	0.3%	114.71	(2.23)
AA	5μg/mL	106.21	(15.29)
RT	20mM	96.69	(16.93)
3NP	1μM	13.31*	(2.49)
AA, RT	5μg/mL, 20mM	95.25	(9.48)
3NP,AA,RT	1μM, 5μg/mL, 20mM	14.23*	(1.97)

Table 1: Effect of specific mitochondrial inhibitors: Complex III (Antimycin A; AA), Complex I (Rotenone; RT), Complex II (3 nitropropionic acid; 3NP), Vehicle (DMSO), or Control (Tris Buffer) on complex II activity. Values represent the percent difference from controls (%) and 1 standard deviation of the mean (STDEV). * Values differ from all other treated groups ($p \leq 0.05$) using a one-way ANOVA.

Densitometric Analysis of Succinate Dehydrogenase Activity in Caudate of treated

mm from Bregma	Treatment	Density (% control)	S.E.M.
+1.7	SALINE	100	2.57
+1.7	METH (10 mg/kg/2h x 4)	111.85	4.11
+1.7	SALINE + 1μM 3-NP *(in vitro)*	84.11*	0.34
+1.7	SALINE + 10mM 3-NP *(in vitro)*	54.28*	0.34
-0.8	SALINE	100	3.29
-0.8	METH (10 mg/kg/2h x 4)	103.50	4.52

Table 2: Assessment of succinate dehydrogenase activity using densitometric analysis of caudate from saline (1ml/kg/2 h X4) and METH (10 mg/kg/2 h X4) treated animals (n=6), or from saline-treated rat slices incubated with 3-NP (1μM or 10 mM). Data are expressed as % control density (arbitrary units) ± S.E.M. * Values different from saline-treated controls ($p \leq 0.05$) using Student's t-test.

Figure legends:

Figure 1: Effect of high-dose METH administration on mitochondrial ETC activity. Columns represent mean enzyme activity of complex I-III (A) or complex II-III (B) in mitochondria isolated from the striata of vehicle- (1ml/kg/2 hr X 4; solid columns) or METH- (10mg/kg/2 h X 4; open columns) treated rats. To examine the effects of METH-induced hyperthermia a third group was added whereby METH-induced hyperthermia was prevented (See Methods; METH-normothermic). All rats were killed 1 h following the last injection. Bars represent 1 S.E.M. for eight animals. * Values different from vehicle-treated controls ($p \leq 0.05$) using one-way ANOVA.

Figure 2: Effect of high-dose METH administration on mitochondrial ETC activity in striatal or hippocampal brain regions. Columns represent enzyme activity of complex II-III in mitochondria isolated from the striatum or hippocampus of vehicle- (1ml/kg/2 h X 4; solid columns) or METH- (4X 10mg/kg/2 h X 4; open columns) treated rats. All rats were killed 1 h following the last injection. Bars represent ± 1 S.E.M. for eight-eleven treated animals. * Values different from vehicle-treated controls ($p \leq 0.05$) using Student's t-test.

Figure 3: Effect of in vitro application of METH on complex II-III activity. Mitochondrial homogenates, isolated from the striatum of untreated rats, were incubated with increasing concentration of METH and complex II-III activity was assessed. Points represent the mean enzyme activity and bars represent 1 S.E.M. for three determinations of enzyme activity using striatal tissue from three different untreated animals.

Figure 4: Effect of high-dose METH on complex II (succinate dehydrogenase) activity in mitochondria isolated from the striatum of treated rats. Rats were treated with either vehicle (1ml/kg/2 h X 4; solid columns) or METH (10mg/kg/2 h X 4; open columns). Rats were killed 1 h after the last injection. Columns represent mean enzyme rate ± 1 S.E.M. for nine-ten treated animals. * Values differ from vehicle-treated group ($p \leq 0.05$) using a Student's t-test.

Figure 5: Role of glutamate receptors in contributing to the METH-induced decreases in complex II activity. Rats were treated with vehicle (1ml/kg/2 h X 4; solid columns) or METH (10mg/kg/2 h X 4; open columns) with or without a post-administration of MK-801 (1mg/kg) or vehicle (1 ml/kg) 1 h and 5 h following the last vehicle or METH administration). All animals were killed 24 h after the last vehicle or METH administration. Columns represent the mean rate of complex II-III activity (in nmoles/min/mg protein) from the striata of treated animals. Bars represent 1 S.E.M. for eight-nine treated animals. * Values different from corresponding controls ($p \leq 0.05$) using one-way ANOVA.

Figure 6: Direct effect of peroxynitrite on complex II-III (A) and I-III (B) enzyme activity. Striatal mitochondrial homogenate was incubated for 3 min with vehicle, 1mM or 0.3μM peroxynitrite. Enzyme activity was assessed as described in Methods and graphed as a percent of control ± S.E.M. * Values different from vehicle-treated controls ($p \leq 0.05$). # Different from the 0.3 μM peroxynitrite concentration using one-way ANOVA ($p \leq 0.05$).

Figure 7: Role of peroxynitrite in contributing to the METH-induced decreases in complex II activity. Rats were pre-treated with saline vehicle (1 ml/kg i.p.) or Fe-TPPS (10mg/kg, i.p. 12 hr and 5 min prior to the first and third METH injection respectively) followed by administration of METH (10ml/kg/2 h X 4) or vehicle (1ml/kg/2 h X 4). All animals were killed 24 h after the last vehicle or METH administration. Columns represent the mean rate of complex II-III activity (in nmoles/min/mg protein) from the striata of treated animals. Bars represent 1 S.E.M. for eight-nine treated animals. * Values different from corresponding controls ($p \leq 0.05$) using one-way ANOVA.

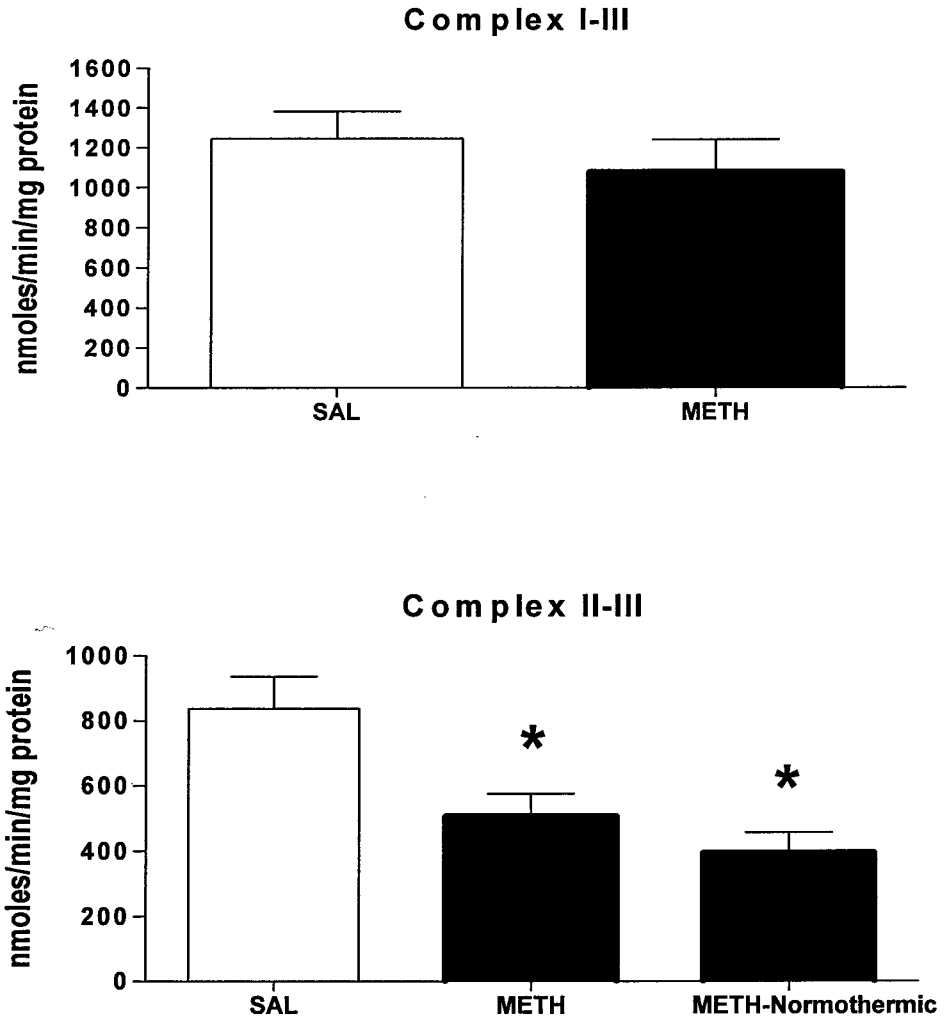
Complex I-III
nmoles/min/mg protein
1600
1400
1200
1000
800
600
400
200
0
SAL
METH
Complex II-III
nmoles/min/mg protein
1000
800
600
400
200
0
*
*
SAL
METH
METH-Normothermic

Figure 1

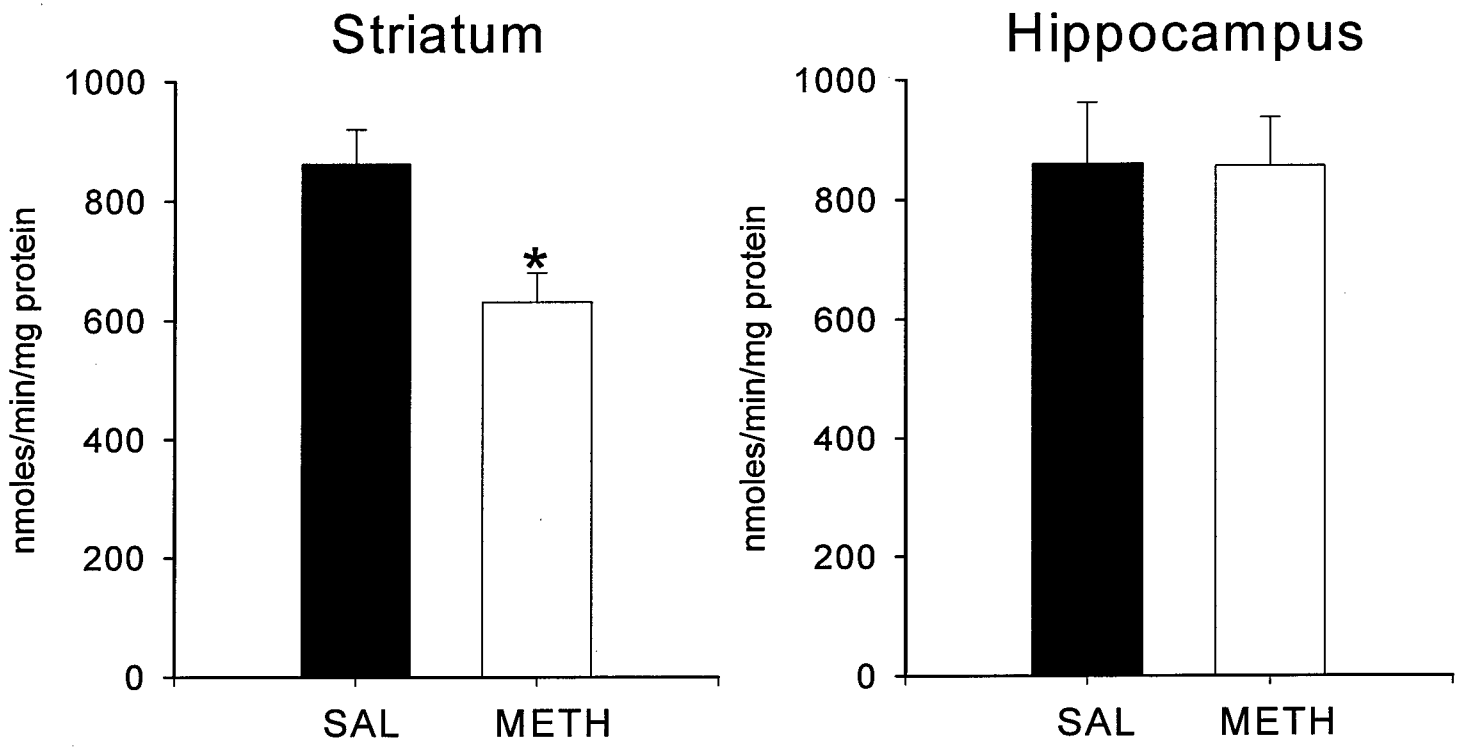
Striatum
Hippocampus
nmoles/min/mg protein
1000
800
600
400
200
0
*
SAL
METH

Figure 2

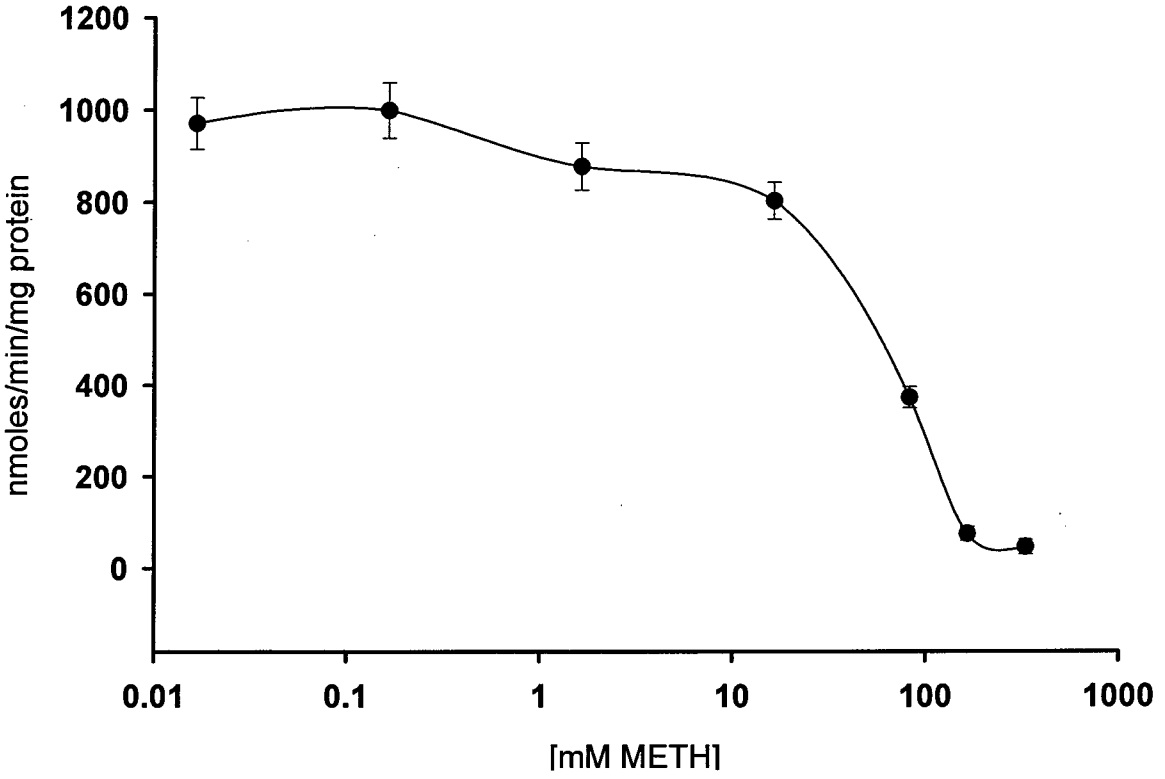
1200
1000
800
600
400
200
0
nmoles/min/mg protein
0.01
0.1
1
10
100
1000
[mM METH]

Figure 3

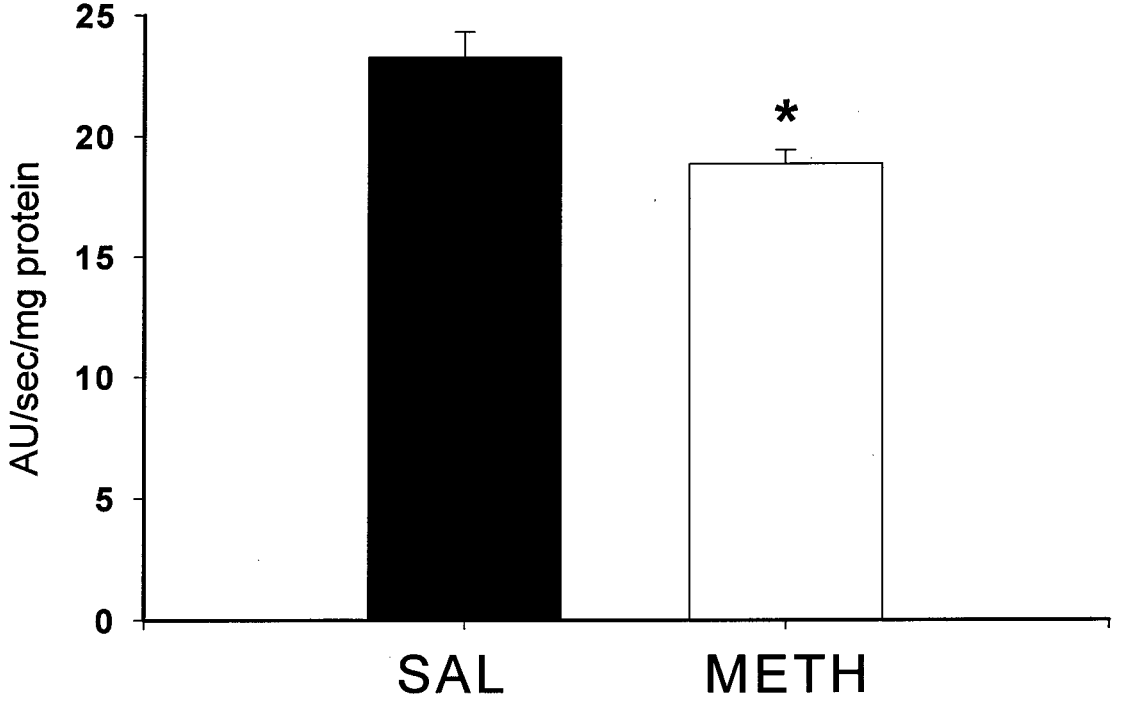

Figure 4

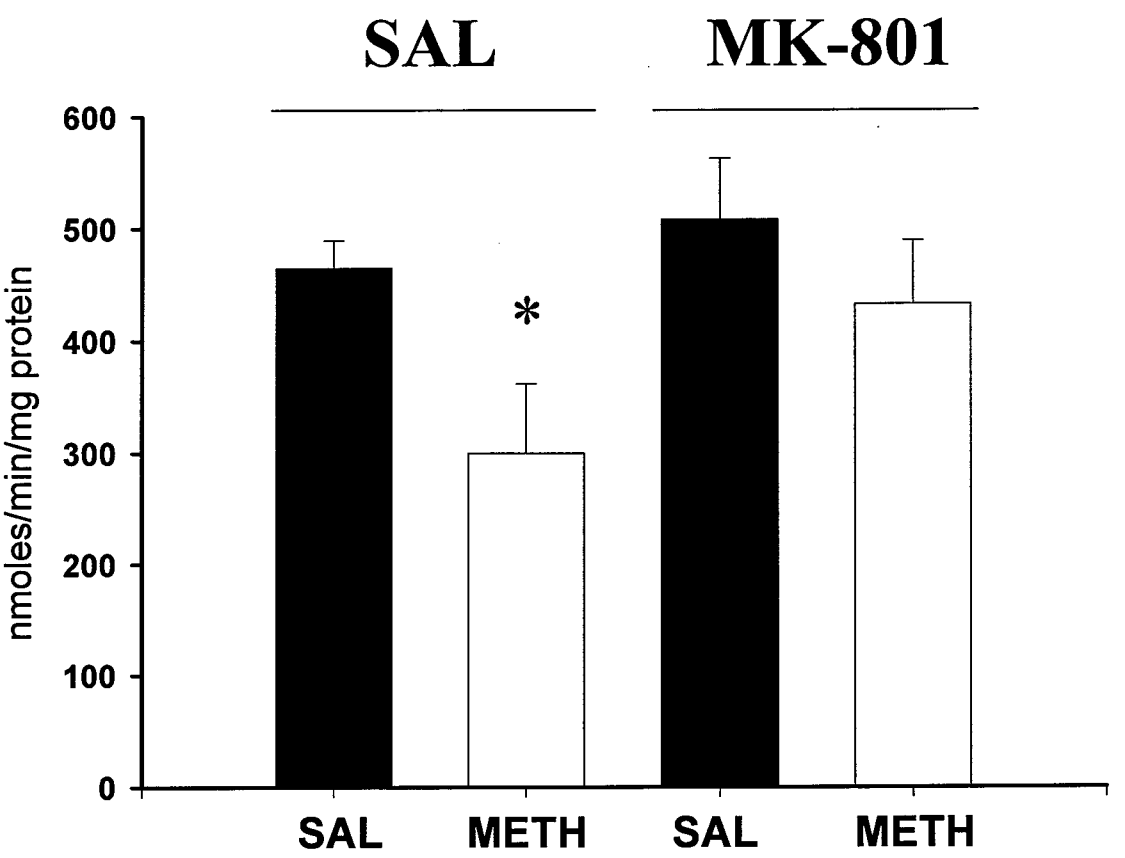
SAL
MK-801
600
500
400
300
200
100
0
nmoles/min/mg protein
*
SAL
METH
SAL
METH

Figure 5

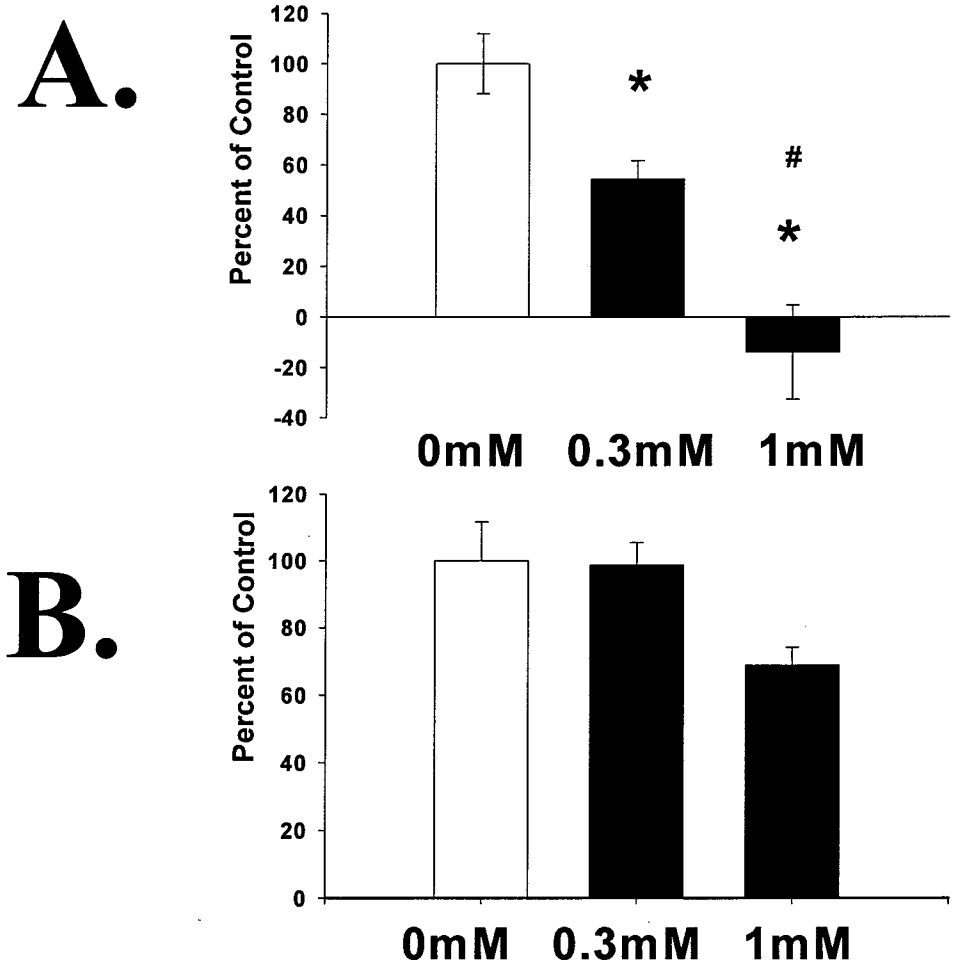
A.
Percent of Control
120
100
80
60
40
20
0
-20
-40
*
#
*
0mM
0.3mM
1mM
B.
Percent of Control
120
100
80
60
40
20
0
0mM
0.3mM
1mM

Figure 6

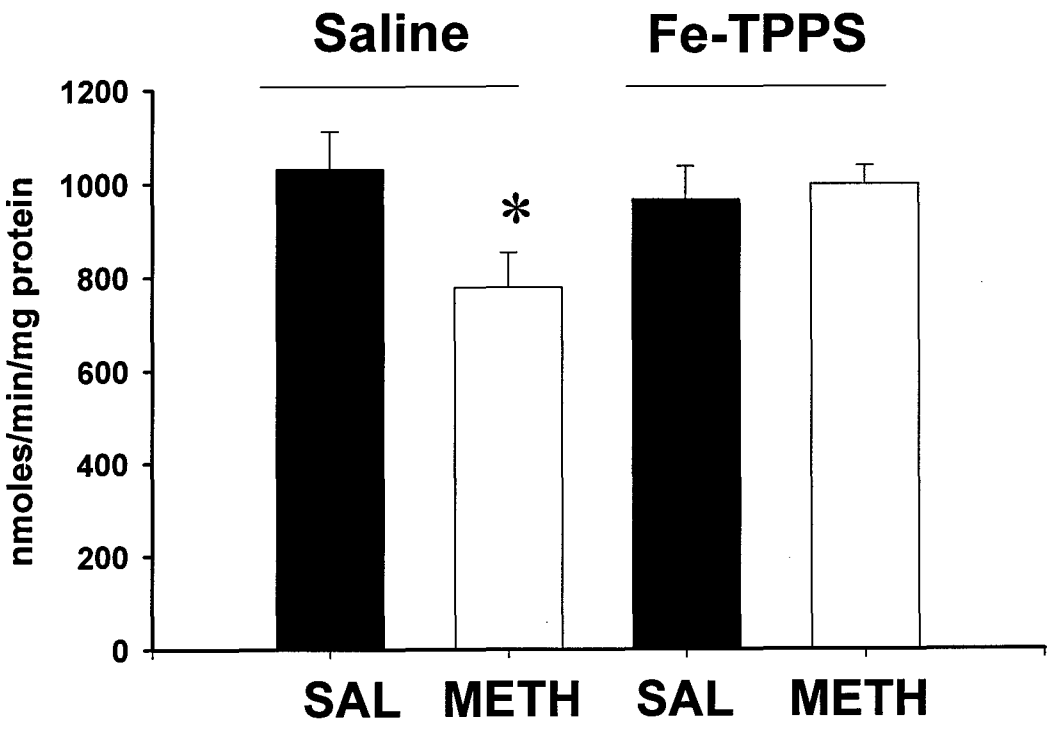
Saline
Fe-TPPS
1200
1000
800
600
400
200
0
nmoles/min/mg protein
*
SAL
METH
SAL
METH

Figure 7

Methamphetamine-Induced Spectrin Proteolysis in the Rat Striatum

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Abstract

Methamphetamine (METH) is a widely abused psychostimulant. Multiple high doses of METH cause long-term toxicity to dopamine (DA) and serotonin (5-HT) nerve terminals in the brain as evidenced by decreases in DA and 5-HT content, decreases in tyrosine and tryptophan hydroxylase activities, decreases in DA and 5-HT reuptake sites, and nerve terminal degeneration. Multiple high-doses of METH are known to elicit a rapid increase in DA release and hyperthermia. Although METH also produces a delayed and sustained rise in glutamate, no studies have shown if METH produces structural evidence of excitotoxicity or have identified the receptors that mediate this toxicity directly, independent of alterations in METH-induced hyperthermia.

These experiments investigated if METH can cause excitotoxicity as evidenced by cytoskeletal protein breakdown in a glutamate receptor-dependent manner. METH increased calpain-mediated spectrin proteolysis in the rat striatum 5 and 7 days after METH administration without affecting caspase-3 dependent spectrin breakdown. This effect was completely blocked with the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist GYKI 52466 but not the NMDA receptor antagonist MK-801. However, AMPA or NMDA receptor antagonism did not attenuate the METH-induced depletions of the dopamine transporter (DAT). Independent mechanisms involved in mediating spectrin proteolysis and DAT protein loss are discussed.

Keywords: Methamphetamine, Excitotoxicity, Glutamate, Spectrin, Calpain

Running Title: Methamphetamine-Induced Spectrin Proteolysis

Abbreviations

5-HT, 5-hydroxytrptamine (serotonin)

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

DA, 3,4-dihydroxyphenylethylamine (dopamine)

METH, methamphetamine

i.p., intraperitoneal

RT, room temperature

SBP, spectrin breakdown product

Introduction

Methamphetamine (METH) is a psychostimulant that is highly abused. Many animal studies support the view that METH is neurotoxic to dopamine (DA) and serotonin (5-HT) terminals. These studies show that METH decreases multiple dopaminergic and serotonergic markers such as: tissue DA and 5-HT content, tyrosine and tryptophan hydroxylase activity, and uptake sites (Gibb and Kogan 1979; Wagner et al. 1980; Bakhit et al. 1981). Moreover, histological and biochemical evidence suggests structural neurodegeneration of DA and/or 5-HT neurons after METH (Gibb and Kogan 1979; Wagner et al. 1980; Bakhit et al. 1981; Ricaurte et al. 1982; Wallace et al. 2003). Despite this evidence, the neurotoxicity of METH remains controversial and the underlying mechanisms mediating damage to DA and 5-HT terminals remain unknown.

METH acutely elevates the extracellular concentrations of DA and glutamate in the striatum (O'Dell et al. 1991; Nash and Yamamoto 1992). It has been hypothesized that increases in both DA and glutamate are necessary for METH toxicity to occur. This is believed to be mediated via the production of oxidative stress and/or glutamate-mediated excitotoxic mechanisms (Stephans and Yamamoto 1994; Fleckenstein et al. 1997a; Fleckenstein et al. 1997b; Yamamoto and Zhu 1998; LaVoie and Hastings 1999). In addition, other studies have shown that glutamate receptor antagonists, or an attenuation of the METH-induced glutamate release, is protective against METH toxicity to DA terminals in the striatum (Sonsalla et al. 1991; Stephans and Yamamoto 1996; Battaglia et al. 2002). However, since hyperthermia is known to contribute to METH-induced damage of DA terminals (Ali et al. 1996), and most glutamate antagonists block the METH-induced hyperthermia (Albers and Sonsalla 1995; Golembiowska et al. 2003),

it is unclear if and how glutamate receptors directly mediate the damage to DA terminals produced by METH.

The increase in glutamate produced by METH (Nash and Yamamoto 1992) can activate multiple glutamate receptors, such as the NMDA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), or metabotropic (Battaglia et al, 2002) receptors but the identification of the glutamate receptors located on DA terminals in the striatum is controversial. Despite findings that intrastriatal NMDA will release DA, such release may not be mediated directly through NMDA receptors located on DA terminals (Keefe et al. 1993) as there is a low level of NMDA receptor expression on striatal DA terminals (Segovia et al. 1997; Betarbet and Greenamyre 1999; Hernandez et al. 2003). It is quite possible that an NMDA-mediated increase in glutamate from corticostriatal terminals could activate other glutamate receptors on DA terminals to release DA, or interact with other neurons in a polysynaptic manner to elevate extracellular DA in the striatum. Although the NMDA receptor antagonist, MK-801, blocks METH-induced decreases in DA content, MK-801 also decreases METH-induced hyperthermia (Sonsalla et al., 1991; Albers and Sonsalla, 1995). Taken together, it is not clear if NMDA receptors have a direct role in mediating the hypothesized excitotoxic effects of METH (Burrows and Yamamoto 2003).

In contrast to the relative paucity of NMDA receptors on DA terminals in the striatum, there appears to be a high level of expression of AMPA receptors (Fan et al. 1999; Dunah et al. 2000; Lai et al. 2003). The GluR1 subunit of AMPA receptors are preferentially expressed in the primate striatum in contrast to the relative absence of the GluR2 subunit (Betarbet and Greenamyre 1999). In light of the findings that the absence

of the GluR2 subunit of the AMPA receptor confers calcium permeability of the AMPA receptor (Hollmann et al. 1991), calcium permeable AMPA receptors may mediate DA release and eventual excitotoxicity to DA terminals.

The cascade of events which follow elevations in intracellular calcium concentrations to produce cell death has been elucidated originally by Siman and co-workers (Siman and Noszek 1988; Siman et al. 1989) as well as by Lynch and colleagues (Seubert et al. 1988a; Seubert et al. 1988b; Lee et al. 1991). Activation of the calcium-dependent protease, calpain, is a primary mechanism contributing to several types of neurodegenerative conditions, including excitatory amino acid-induced neurotoxicity associated with traumatic brain injury, ischemia, and hyperthermia (Morimoto et al. 1997; Pike et al. 1998; Buki et al. 1999). Calpain specifically degrades the cytoskeletal membrane protein, spectrin, into 145 and 150 kDa breakdown products (Harris and Morrow 1988). Caspase-3 is a another cysteine protease which is exclusively involved in apoptotic pathways and also degrades spectrin but produces a 120 kDa spectrin fragment (Wang 2000). Based on this, we hypothesize that METH-induced glutamate release activates calcium permeable AMPA receptors and calcium-dependent proteases such as calpain and caspase-3, to proteolytically cleave the cytoskeletal scaffolding protein, spectrin.

The experiments described herein assessed the temperature-independent roles of NMDA and AMPA receptors in mediating the hypothesized excitotoxic structural damage in the rat striatum as evidenced by the proteolysis of spectrin produced by METH.

Materials and Methods

Animals

Male Sprague Dawley rats weighing between 240-300 g (Harlan Sprague Dawley, Indianapolis, IN) were used in all experiments. Rats were housed 3/cage, provided with food and water ad libitum and kept in a temperature-controlled environment (20-22 °C) on a 12-hr light/dark cycle. All experiments were performed with strict accordance to the Nationals Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drugs and Drug Administration

D-Methamphetamine, Dulbecco's solution and GYKI 52466 were all purchased from Sigma Aldrich (St. Louis, MO). MK-801 was acquired from Tocris (Ellisville, MO). All injections were administered intraperitoneally (i.p.). The dosing paradigm was 10 mg/kg METH or 0.9% saline (1 ml/kg) every 2 hrs for a total of 4 injections. GYKI 52466 (10 mg/kg) and MK-801 (1 mg/kg) were injected i.p. 15 min prior to each METH or saline injection. GYKI 52466 (1ml/kg) was dissolved in vehicle containing 100 µl 0.1N HCl, 400 µl Dulbecco's phosphate buffered saline (pH 7.4), and 500 µl dH_2O. MK-801 was dissolved in 0.9% saline.

Western Analysis for Striatal DAT and Spectrin Breakdown Products

Rats were killed 3, 5 or 7 days after the final METH or saline injection by rapid decapitation. Brains were removed and striata were dissected and frozen on dry ice. Striata were then stored at -80 °C until assayed.

The striatum was analyzed for spectrin breakdown products (SBPs) as previously described (Zhang et al. 2002). Briefly, the striatum was homogenized in 20:1 vol/wt (μl/mg) buffer containing 10 mM Tris (pH 7.4), 10 mM EGTA, 250 mM sucrose, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin A, and 1 mM PMSF (Sigma Aldrich, St. Louis, MO). Protein concentrations were determined by the method of Bradford (Bio-Rad, Hercules, CA). Samples were prepared for electrophoresis by diluting the homogenates 1:1 with 2X SDS loading buffer (Invitrogen, Grand Island, NY) and heated to 85 °C for 5 min.

Samples (10 μg) were electrophoresed in an Invitrogen gel system and later transferred onto PVDF membranes. Spectrin and dopamine transporter (DAT) analyses were performed utilizing 6% and 10% Tris-glycine gels, respectively. Membranes for SBPs were rinsed in TBS-T (20 mM Tris (pH 7.6), 137 mM NaCl, 0.1% Tween 20) and blocked for 1 h at room temperature (RT) in TBS-T containing 5% powdered milk. Membranes for DAT protein levels were rinsed in TBS and blocked for 1 h at RT with TBS containing 0.5% Tween and 5% powdered milk. Membranes were then probed with primary antibody for non-erythroid α-spectrin (MAB1622, Chemicon, 1:5000) or DAT (SC-1433, Santa Cruz, 1:2500) overnight at 4 °C. The following day, membranes with spectrin and DAT were washed with TBS-T or TBS, respectively, and probed with the appropriate anti-mouse or anti-goat HRP-linked IgG antibody (Amersham Pisctaway, NJ; Santa Cruz Biotechnology Santa Cruz, CA) for 1 h at RT. Spectrin and DAT membranes were washed again and visualized using chemiluminescence reagents and Hyperfilm (Amersham, Pisctaway, NJ). The densities of SBPs at 120 and 145 kDa (Wang 2000)

and DAT protein levels were quantified on a Kodal Kodak Gel Logic 100 imaging system.

Temperature Measurements

The ambient temperature was maintained at 22-23 °C for all experiments. The cage tops were removed prior to each experiment so that the rats did not become lethally hyperthermic. The core body temperature of the rats was measured 1 h after each METH or saline injection by a rectal probe digital thermometer (Thermalert TH-8; Physitemp Instruments, Inc., Clifton, NJ). Since MK-801 can attenuate METH-induced hyperthermia, hyperthermia was maintained to assess the temperature independent effects of MK801 by leaving the cage filter tops on the cages in which the rats were treated.

Statistics

All statistical analyses were performed using either two-way ANOVA with post hoc Tukey's tests, Student's *t* tests or the Pearson Product Moment test to assess differences or correlations between groups. Saline controls were run in parallel with METH treated rats for each time point and the appropriate comparisons were made between each group. All statistics were determined using Sigma Stat 2.03. Statistical significance was set at $p < 0.05$. Data are presented as percent of saline or vehicle control with means ± SEM.

Results

METH Induces Spectrin Proteolysis and DAT Protein Depletions in the Striatum

Figure 1 indicates the temporal profile of spectrin proteolysis following neurotoxic doses of METH in the striatum. There is a significant time ($F_{(1,60)}$=3.538, $p<0.05$), treatment ($F_{(1,60)}$=11.365, $p<0.001$) and time X treatment interaction ($F_{(2,60)}$=3.538, $p<0.05$) effect with regards to calpain-mediated spectrin proteolysis after a neurotoxic METH regimen. Post-hoc analysis indicated that calpain-mediated spectrin proteolysis is significantly increased by 78 ± 18 and 35 ± 18% above control levels, 5 and 7 days after METH administration ($p<0.05$), respectively. There is also a significant decline in spectrin proteolysis from 5 to 7 days after METH treatment ($p<0.05$). There was no significant increase in spectrin proteolysis 3 days after METH and no change in caspase-3 dependent spectrin proteolysis, as indicated by the 120 kDa SBP, at 3, 5 or 7 days following METH administration.

Figure 2 illustrates that DAT is significantly decreased over time with an overall treatment effect ($F_{(1,60)}$=116.669, $p<0.001$) at all three time points tested. DAT immunoreactivities after METH treatment are 27 ± 8, 20 ± 5 and 38 ± 5% of control values for the 3, 5 and 7 day time points respectively.

Figure 3 shows that the degree of calpain-mediated spectrin proteolysis correlates with overall METH-induced DAT depletions in the striatum. This correlation was significant only at the 5 day time point ($r=0.737$, $p<0.01$).

AMPA Antagonism Blocks METH-Induced Spectrin Proteolysis

Figure 4 illustrates the effects of the AMPA antagonist GYKI 52466 on METH-induced spectrin proteolysis and DAT depletions in the striatum. There is a significant interaction between groups for spectrin proteolysis ($F_{(1,37)}$=10.031, $p<0.01$) such that GYKI 52466 significantly blocked METH-induced spectrin proteolysis ($p<0.05$) but did not affect spectrin proteolysis in saline controls. DAT immunoreactivity was decreased after METH ($F_{(1,38)}$=37.034, $p<0.001$), but this decrease was not blocked by GYKI 52466.

Figure 5 shows that METH significantly increases core body temperature ($F_{(1,38)}$=110.760, $p<0.001$) but these increases were not attenuated by GYKI 52466.

MK-801 Does Not Effect METH-Induced Spectrin Proteolysis or DAT Depletions

Figures 6 and 7 demonstrate the effects of the NMDA antagonist MK-801 on METH-induced spectrin proteolysis, DAT depletions, and hyperthermia. Student's *t* tests indicate that METH increases spectrin proteolysis in the striatum ($p<0.05$), but this effect is not attenuated by MK-801 (Figure 6A). METH also decreased DAT immunoreactivity in the striatum ($p<0.001$), however the depletion was not attenuated by MK-801 (Figure 6B). Because MK-801 is known to block the METH-induced hyperthermia, efforts were made to maintain similar levels of hyperthermia in all rats to test the temperature independent effects of MK-801 on METH-induced spectrin proteolysis. Figure 7 illustrates that there is no difference in hyperthermia between rats who received METH with or without MK-801.

Discussion

The present results illustrate that METH causes calpain-mediated spectrin proteolysis in a time-dependent manner within the striatum. The increases in spectrin breakdown products also correlate with METH-induced DAT depletions 5 days following a neurotoxic METH regimen. The AMPA antagonist GYKI 52466, but not the NMDA antagonist MK-801, blocked METH-induced spectrin proteolysis in the striatum and did so in a temperature-independent manner. However, neither antagonist attenuated the DAT depletions observed following METH administration. It is suggested that METH-induced increases in extracellular glutamate (Nash and Yamamoto 1992) selectively activate AMPA receptors on DA terminals in the striatum to produce calpain-mediated spectrin proteolysis. However, the mechanisms underlying glutamate-mediated spectrin proteolysis appear to be independent of the mechanisms by which METH decreases DAT protein in the striatum.

The increase in calpain-mediated spectrin proteolysis appeared at both 5 and 7 days but not at 3 days after METH administration. Furthermore, the increase at 7 days was significantly less than the spectrin proteolysis observed at 5 days. This time course of spectrin proteolysis is in accordance with the time course of silver staining in the striatum after a multiple dosing METH regimen (Ricaurte et al., 1982). The late onset of spectrin degradation could be explained through the delayed yet sustained elevation of glutamate in the striatum during a neurotoxic METH regimen (Nash and Yamamoto 1992; Abekawa et al. 1994) and the time needed for the accumulation and detection of SBPs. Thus, persistent stimulation of glutamate receptors may be necessary to sufficiently

increase the intracellular concentrations of calcium and calpain activity to degrade the terminal and permit the detection of accumulated SBPs.

The decline in immunoreactivity for the 145 kDa SBP at 7 days can be explained by the removal of spectrin fragments from the CNS following terminal degradation. It also has been shown that SBPs are cleared from the CNS and transported to the cerebrospinal fluid after global ischemia (Pike et al. 2001; Pike et al. 2004). Other studies have shown that spectrin proteolysis reaches a maximum level after 3 days following an ischemic episode or traumatic brain injury conditions that then declines over time (Beer et al. 2000; Zhang et al. 2002).

Spectrin can be proteolyzed by both calpain and caspase-3 (Siman and Noszek 1988;Wang et al. 1998). Calpain degrades spectrin to the 145 kDa SBP under necrotic and apoptotic conditions, while caspase-3 lyses spectrin into a 120 kDa SBP exclusively under apoptotic conditions (McGinnis et al. 1998; Zhao et al. 1999; Lu et al. 2002). The present results showing an increase in the 145 kDa SBP over time after METH without a change in the 120 kDa SBP suggest that METH-induced spectrin proteolysis is mediated only through calpain activation and not through an increase in caspase-3 activity.

METH is toxic largely to DA and 5-HT terminals in the striatum and not their corresponding cell bodies (Ricaurte et al. 1982; Seiden et al. 1988). Apoptosis generally involves DNA damage and nuclear shrinkage. Since neuronal terminals do not contain DNA or a nucleus, it would be expected that necrotic and not apoptotic mechanisms would be responsible for DA terminal damage after METH administration. The data described here suggests that striatal spectrin is degraded exclusively by calpain and not caspase-3. Considering that caspase-3 is a pro-apoptotic factor, the lack of caspase-3

dependent proteolysis is consistent with the conclusion that terminal degeneration by METH is mediated by necrotic and excitotoxic mechanisms. In contrast, Cadet and colleagues have stated that both caspase-3 and calpain are activated for several days within striatal GABA cells of the mouse following a single, high-dose injection of METH (Jayanthi et al. 2004). The discrepancy between those results and the data shown here could be accounted for through the differences in dosing paradigm and the animal species. There are many different proteins which are substrates for caspase-3. It may be possible that caspase-3 is activated after METH treatment but cleaves a substrate other than spectrin. Therefore, the METH-induced increase in extracellular glutamate (Stephans and Yamamoto, 1994) may mediate spectrin proteolysis in DA terminals and not in GABA cells.

The AMPA receptor glutamate antagonist GYKI 52466, but not the NMDA receptor glutamate antagonist MK-801, blocked the METH-induced spectrin proteolysis in a temperature-independent manner. While spectrin proteolysis can been attenuated by either an AMPA and NMDA antagonist (Le Peillet et al. 1992; Block et al. 1996; Minger et al. 1998), it appears that METH-induced striatal spectrin proteolysis is selectively mediated through the AMPA receptor. Many pharmacological agents, including glutamate antagonists, have been shown to block hyperthermia caused by METH. Thus their protective effects against toxicity to DA terminals can be attributed to the attenuation of hyperthermia (Bowyer et al. 1994; Albers and Sonsalla 1995). Since hyperthermia can exacerbate spectrin proteolysis (Morimoto et al. 1997; Kitagawa et al. 1999), it is possible that the protective effect of AMPA antagonism was due to the attenuation of METH-induced hyperthermia. However, since GYKI 52466 did not

attenuate METH-induced hyperthermia, the inhibition of spectrin proteolysis by AMPA receptor blockade is temperature independent.

Although GYKI 52466 attenuates METH-induced spectrin proteolysis, it is unclear if the protective effect of the AMPA antagonist is through a direct blockade of AMPA receptors located on DA terminals or through an indirect decrease in the extracellular concentrations of glutamate. Microdialysis experiments have shown that GYKI 52466 does attenuate the rise in striatal glutamate during ischemia (Arvin et al. 1994). It is possible that GYKI 52466 decreases the METH-induced elevations of extracellular glutamate to ultimately attenuate spectrin proteolysis. While the present findings are supportive of the conclusion that GYKI 52466 may be acting directly at AMPA receptors located on DA terminals, future studies investigating if AMPA antagonism affects METH-induced increases in extracellular glutamate are warranted.

Microdialysis studies have indicated that AMPA receptors mediate DA release in the striatum (Ohta et al. 1994; Sakai et al. 1997; Segovia et al. 1997; Hernandez et al. 2003). These AMPA receptors appear to lack the GluR2 subunit (Betarbet and Greenamyre 1999; Lai et al. 2003) and demonstrate calcium permeability (Hollmann et al. 1991). Therefore, a massive influx of calcium could activate calpain within the DA terminal and thereby degrade the cytoskeleton through proteolysis of spectrin. In contrast, there is a paucity of evidence for NMDA receptor expression on DA terminals in the striatum (Keefe et al. 1993; Dunah et al. 2000; Lai et al. 2003). Our finding that MK-801 was not effective at decreasing METH-induced spectrin proteolysis when rats were maintained at hyperthermic temperatures is consistent with those studies.

It is interesting to note that neither glutamate antagonist attenuated the METH-induced DAT depletions 5 days after the neurotoxic regimen. There are two possible explanations for the dissociation between the inability of the antagonist to attenuate the decrease in DAT protein while blocking the increase in spectrin proteolysis: 1) The mechanisms underlying spectrin proteolysis and DAT depletions could be independent of one another yet both effects are localized to DA terminals in the striatum or 2) spectrin proteolysis is not localized to DA terminals but to some other neuronal subtype in the striatum. The first possibility could be explained by the fact that while both oxidative stress and glutamate excitotoxicity have been implicated in METH-induced damage to DA terminals, (Wagner et al. 1985; Yamamoto and Zhu 1998; LaVoie and Hastings 1999; Harold et al. 2000; Itzhak et al. 2000), excitotoxicity could occur independent of decreases in DAT protein produced by oxidative stress. Likewise, oxidative stress could target DAT compared to the excitotoxic effects to the cytoskeleton. Thus, even in the face of diminished cytoskeletal proteolysis with an AMPA antagonist, oxidative damage to DAT could still persist.

A second possibility is that spectrin proteolysis does not occur in DA terminals. A likely target is the 5-HT terminal. Striatal 5-HT terminals are damaged by systemic METH administration and 5-HT is released from these neurons following elevations of extracellular glutamate or the perfusion of NMDA into the striatum (Hotchkiss et al. 1979; Bakhit et al. 1981; Schmidt and Gibb 1985; Green et al. 1992; Ohta et al. 1994; Abellan et al. 2000). It is possible that spectrin proteolysis and loss of serotonergic terminals mediated through glutamatergic mechanisms is also correlated 5 days after a neurotoxic METH regimen. If glutamate receptor antagonism attenuates 5-HT toxicity in a

temperature-independent manner, the mechanisms that lead to damage of 5-HT terminals in the striatum could be different from those mediating damage to DA terminals. This potential outcome may indicate a differential susceptibility of DA versus 5-HT neurons to glutamate-mediated excitotoxicity.

Other targets can be damaged by glutamate receptor mediated mechanisms. GABA or acetylcholine (ACh) neurons could also be destroyed by METH-induced increases in glutamate transmission. There is some evidence that substance P containing neurons, but not neurons that express enkephalin, may be damaged by METH (Chapman et al. 2001). Cadet and colleagues have also indicated that apoptotic events occur in the GABA cells of mice after METH treatment (Jayanthi et al. 2004). However, an earlier study by Hotchkiss and Gibb (1979) indicated no change in striatal glutamic acid decarboxylase or choline acetyltransferase activities several days after METH administration. Considering that there is a significant correlation between a decrease in DAT protein and an increase in calpain-mediated SBPs after METH (Figure 3), it seems unlikely that spectrin degradation is occurring in neurons other than DA or 5-HT terminals in the striatum.

In summary, this is the first evidence that a neurotoxic METH regimen can produce structural damage to neurons in an excitotoxic manner that involves AMPA receptors and calpain activation. In contrast, AMPA or NMDA receptors do not appear to directly mediate the decreases in striatal DAT protein produced by METH. Therefore, the mechanisms underlying membrane degradation and damage to the dopamine transporter appear to be independent of one another. Considering that calpain activation has been implicated in Parkinson's disease, Huntington's disease and Alzheimer's

disease, future studies that elucidate the specific neuronal phenotype undergoing METH-induced spectrin proteolysis are warranted.

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Figure Legends

Figure 1A-B. Time-dependent effects of METH on spectrin proteolysis. Animals were killed 3, 5 or 7 days after the final METH or saline injection. Striata were homogenized and analyzed for spectrin breakdown products (SBPs) at 145 and 120 kDa. Data are represented as means ± SEM percentage of control values. The Control group is representative of saline rats killed 5 days after injection and not all Control groups were shown for simplicity. **(A)** Effects of METH on the 145 kDa SBP. * indicates that spectrin proteolysis is increased ($p<0.05$) above controls both at 5 and 7 days after the final METH injection. # indicates that spectrin proteolysis at 7 days is significantly less than at 5 days ($p<0.05$). **(B)** Effect of METH on the 120 kDa SBP. The 120 kDa SBP is not significantly different from control levels at any time-point. n=4, 12 and 11 for the 3, 5 and 7 day time-points respectively. n=16 for the Saline Control group. Samples of representative blots of the 145 and 120 kDa SBP bands are illustrated at the bottom of the graphs.

Figure 2. Time-dependent effects of METH on striatal dopamine transporter (DAT) immunoreactivity measured by western blot analysis. Rats were killed 3, 5 or 7 days after the last METH or saline injection. Data are means ± SEM percentage of control values. The Control group is representative of saline rats killed 5 days after injection. Not all Control groups were shown for simplicity. * illustrates DAT levels from METH treated rats are significantly decreased from control values ($p<0.001$). n=4, 12 and 11 for the 3, 5 and 7 day time-points respectively. n=16 for the Saline Control group. Samples of representative blots of the 78 kDa DAT band are illustrated at the bottom of the graph.

Figure 3. Relationship between changes in DAT density and the 145 kDa SBP. Rats were killed 5 days after the last METH injection and striatal tissue was homogenized and analyzed for DAT protein and SBPs at 145 kDa. The Pearson Product Moment test indicated a significant negative correlation between DAT levels and calpain-mediated spectrin proteolysis ($r=0.737$; $p<0.01$). $n = 16$ rats.

Figure 4A-B. Effect of the AMPA antagonist, GYKI 52466 (10 mg/kg, i.p.) on METH_induced changes in **(A)** 145 kDa SBP and **(B)** dopamine transporter immunoreactivity. All rats were killed 5 days after the final METH or saline injection and analyzed for calpain-mediated SBPs and DAT protein. Data are represented as means ± SEM percentage of control values. **(A)** METH alone increases spectrin proteolysis and this effect is blocked by GYKI 52466. GYKI 52466 itself has no effect on spectrin proteolysis. * designates a significant difference from Vehicle/Saline ($p<0.05$). # represents a significant difference from Vehicle/METH ($p<0.05$). **(B)** METH significantly decreases striatal DAT protein ($p<0.001$) but GYKI 52466 does not attenuate the METH-induced DAT depletion in the striatum. GYKI 52466 alone does not alter striatal DAT protein. * indicates a significant decrease from the Vehicle/Saline group. $n = 9$-12 for all treatment groups. Samples of representative blots of the 145 kDa SBP and 78 kDa DAT bands from each group are illustrated at the bottom of the graphs.

Figure 5. Rectal body temperatures after METH and/or GYKI 52466. Vehicle or GYKI 52466 (10 mg/kg, i.p.) was injected 15 min prior to each METH (10 mg/kg, i.p.) or saline

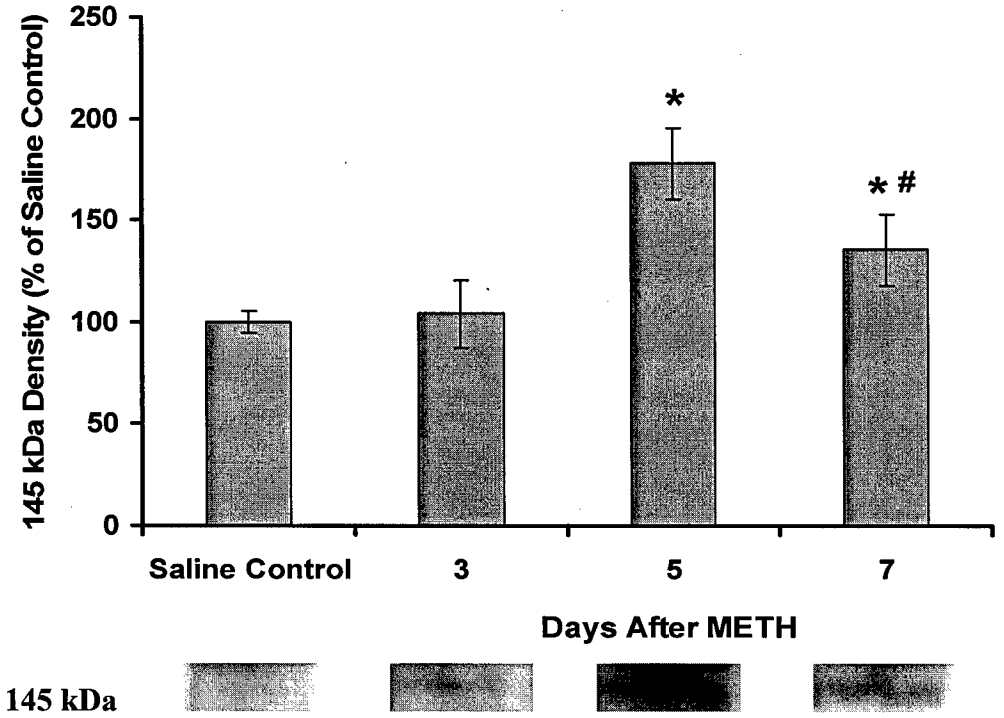
injection. The average for the 4 temperature readings for each individual rat was then calculated. Data are means ± SEM percentage of control values. METH increases core body temperature and GYKI 52466 does not attenuate METH-induced hyperthermia. * indicates a significant difference ($p<0.001$) from the Vehicle/Saline group. $n = 9$-12 for all treatment groups.

Figure 6A-B. Effect of the NMDA antagonist, MK801 (1 mg/kg, i.p.) on METH_induced changes in **(A)** 145 kDa SBP and **(B)** dopamine transporter (DAT) immunoreactivity. Saline or MK-801 was injected 15 min prior to each METH (10 mg/kg, i.p.) or saline injection. METH or saline was administered every 2 hrs for a total of 4 injections. All rats were killed 5 days after the final METH or saline injection and analyzed for calpain-mediated SBPs and DAT protein. Data are means ± SEM percentage of control values. **(A)** The 145 kDa SBP was increased by METH alone ($p<0.05$). Comparisons between the METH rats that received either saline or MK-801 indicate that MK-801 does not attenuate the METH-induced spectrin proteolysis ($p>0.10$). * illustrates a significant increase from the Saline/Saline group ($p<0.05$). **(B)** METH decreases striatal DAT protein ($p<0.001$) but prior administration of MK-801 (MK-801/METH) does not attenuate the METH-induced DAT depletion in the striatum ($p>0.10$) when compared to Saline/METH rats. * indicates a significant decrease from the Saline/Saline group. $n = 8$ for all treatment groups. Samples of representative blots of the 145 kDa SBP or 78 kDa DAT bands are illustrated at the bottom of the graphs.

Figure 7. Rectal body temperatures after METH and/or MK-801. Saline or MK-801 (1 mg/kg, i.p.) was injected 15 min prior to each METH (10 mg/kg, i.p.) or saline injection. METH or saline was injected every 2 hours for a total of 4 injections. Temperatures were taken 1 h after each METH or saline injection. The average of the 4 temperature readings for each individual rat was then calculated. Data are represented as means ± SEM percentage of control values. Student's *t* tests indicate that both METH treatment groups have a significant rise in core body temperature compared to controls ($p<0.001$). There is no statistically significant difference between the two METH groups ($p=0.382$). * illustrates a significant difference ($p<0.001$) from the Saline/Saline group. $n = 8$ for all treatment groups.

Fig. 1

A


145 kDa Density (% of Saline Control)
250
200
150
100
50
0
*
* #
Saline Control
3
5
7
Days After METH
145 kDa

B

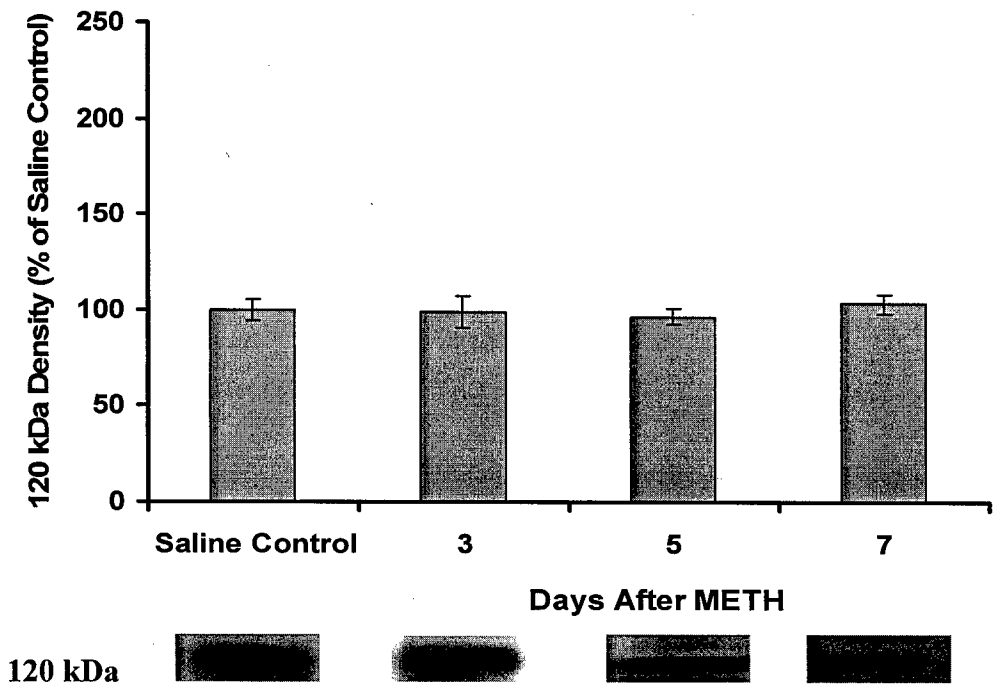

120 kDa Density (% of Saline Control)
250
200
150
100
50
0
Saline Control
3
5
7
Days After METH
120 kDa

Fig. 2

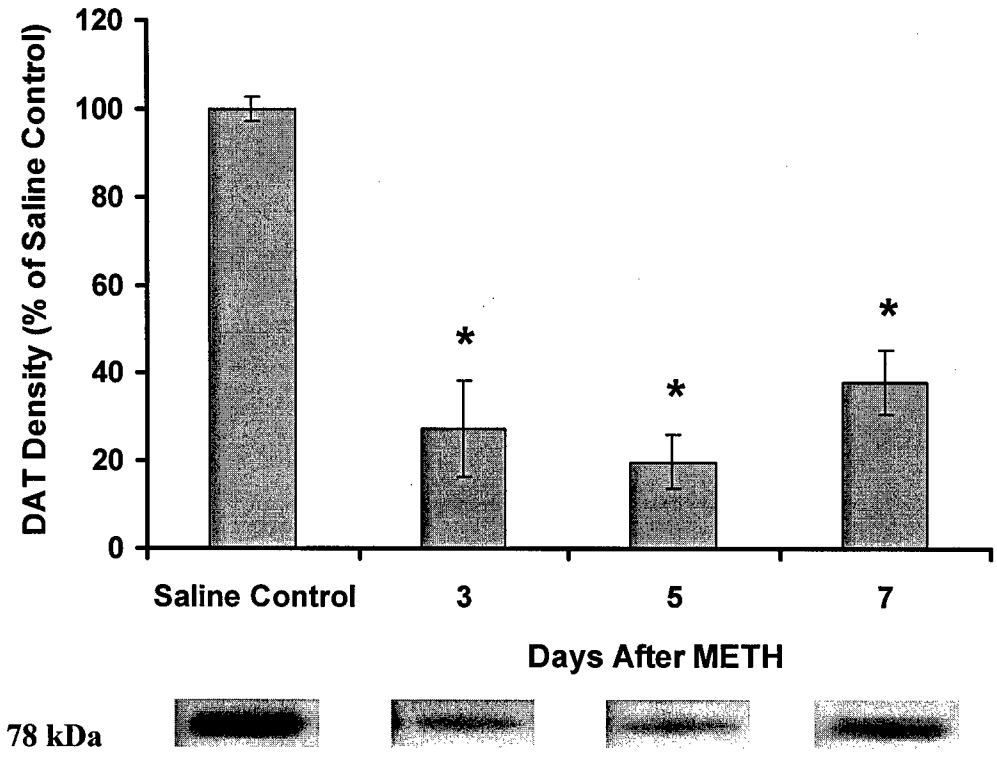

DAT Density (% of Saline Control)
120
100
80
60
40
20
0
*
*
*
Saline Control
3
5
7
Days After METH
78 kDa

Fig. 3

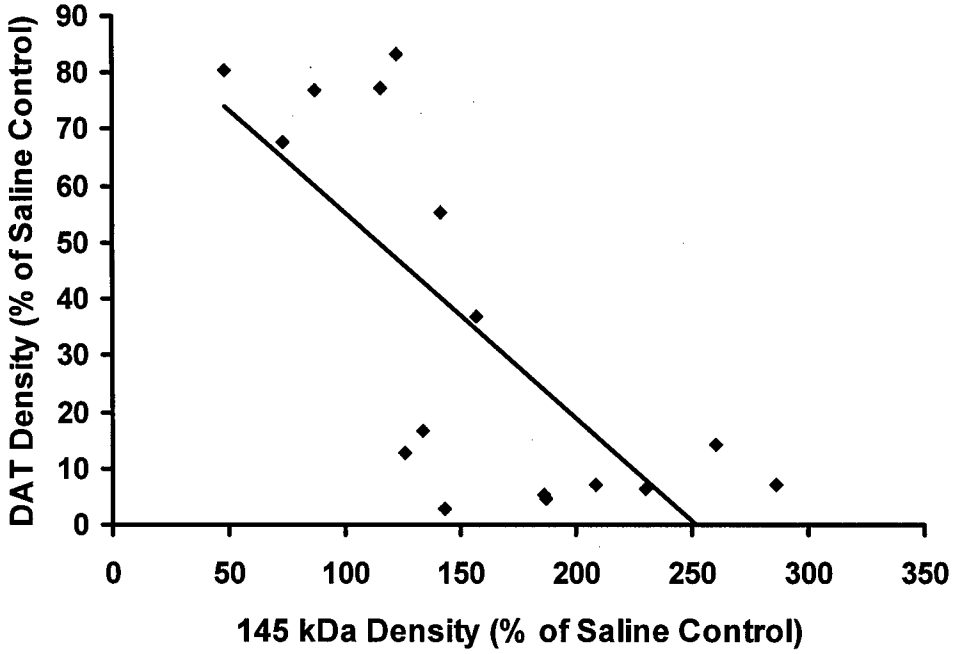
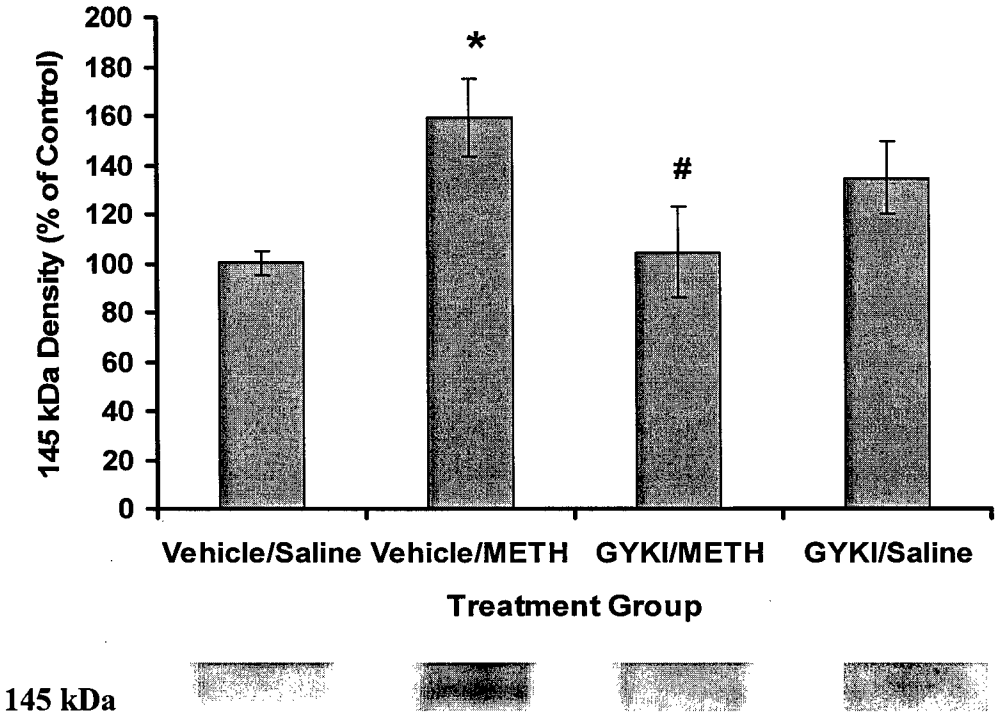

DAT Density (% of Saline Control)
90
80
70
60
50
40
30
20
10
0
0
50
100
150
200
250
300
350
145 kDa Density (% of Saline Control)

Fig. 4

A


145 kDa Density (% of Control)
200
180
160
140
120
100
80
60
40
20
0
*
#
Vehicle/Saline
Vehicle/METH
GYKI/METH
GYKI/Saline
Treatment Group
145 kDa

B

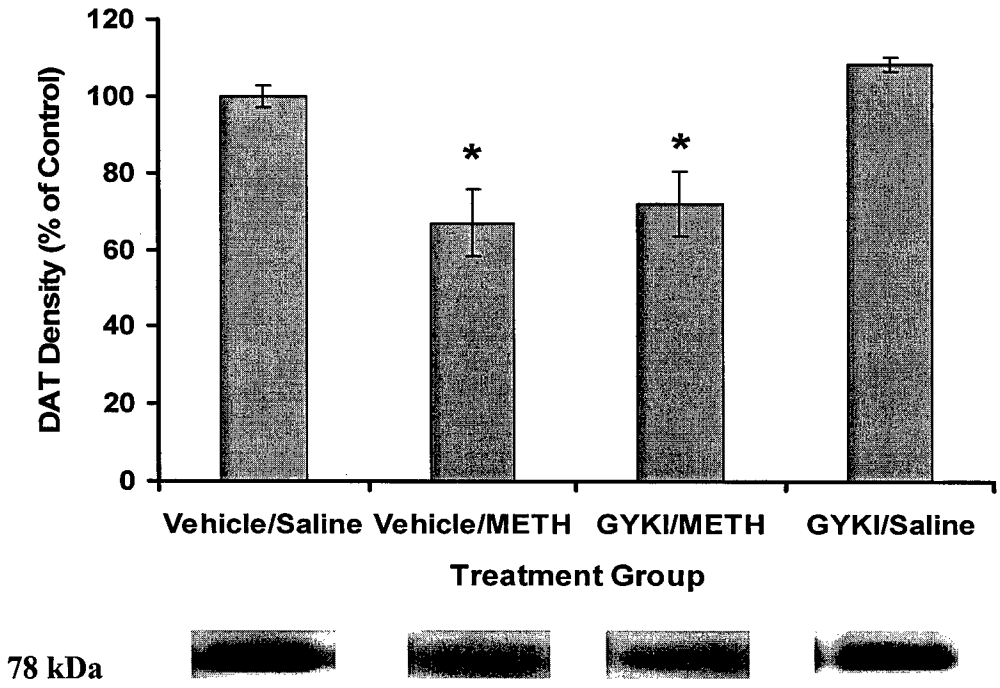

DAT Density (% of Control)
120
100
80
60
40
20
0
*
*
Vehicle/Saline
Vehicle/METH
GYKI/METH
GYKI/Saline
Treatment Group
78 kDa

Fig. 5

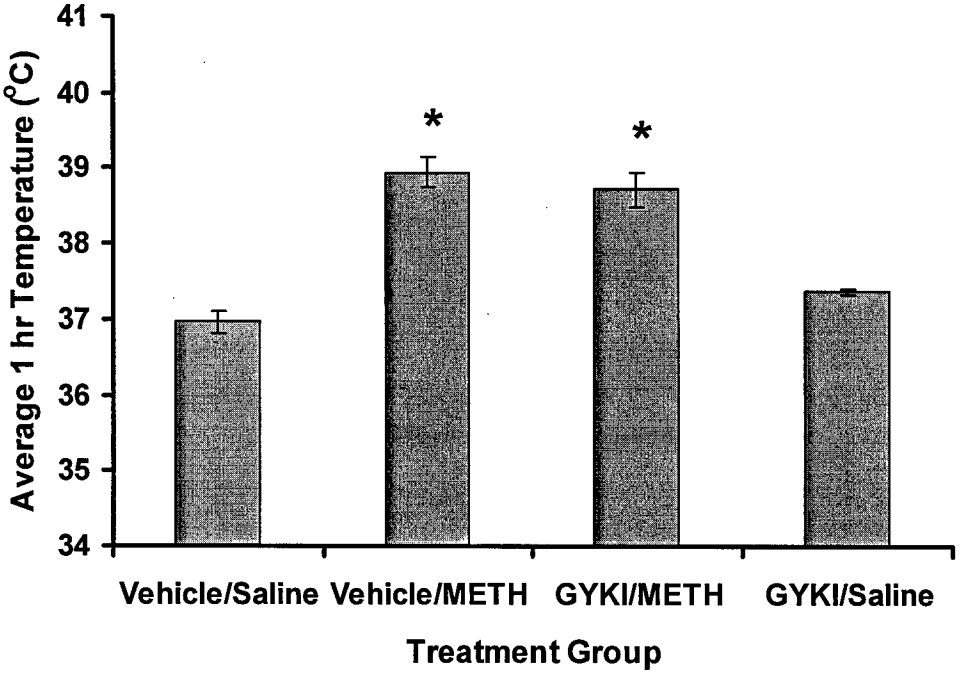
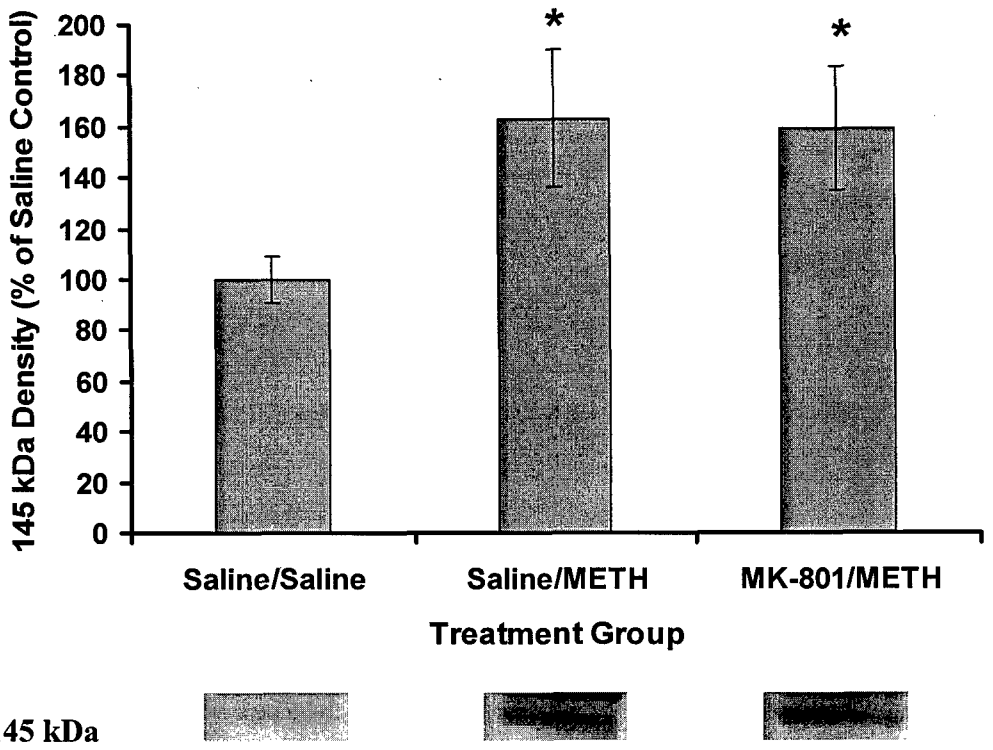

41
40
39
38
37
36
35
34
Average 1 hr Temperature (°C)
*
*
Vehicle/Saline
Vehicle/METH
GYKI/METH
GYKI/Saline
Treatment Group

Fig. 6

A


145 kDa Density (% of Saline Control)
200
180
160
140
120
100
80
60
40
20
0
*
*
Saline/Saline
Saline/METH
MK-801/METH
Treatment Group
145 kDa

B

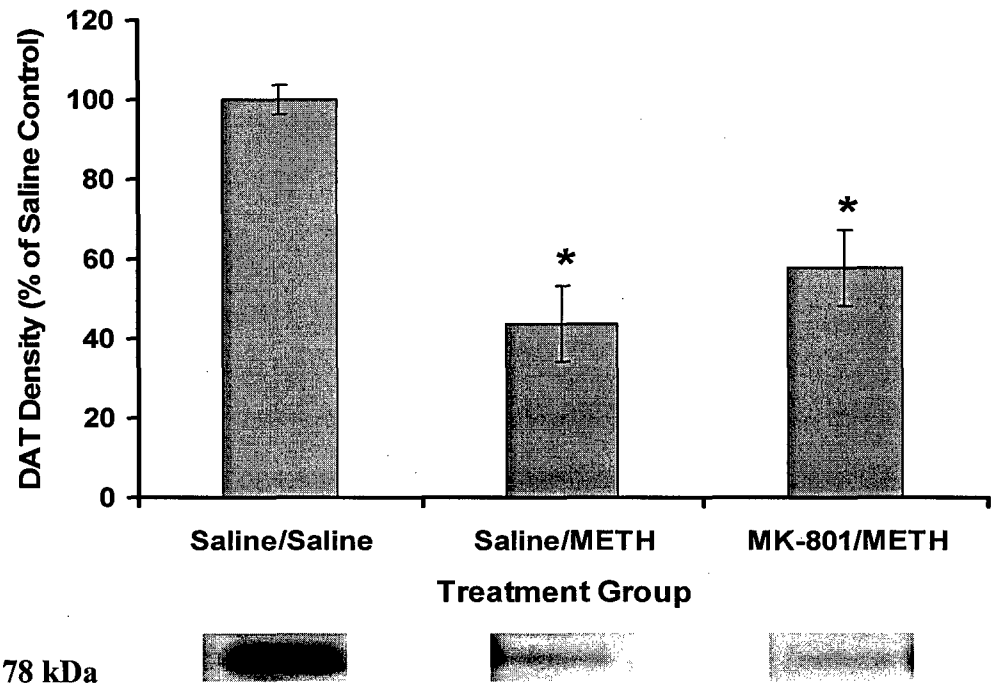
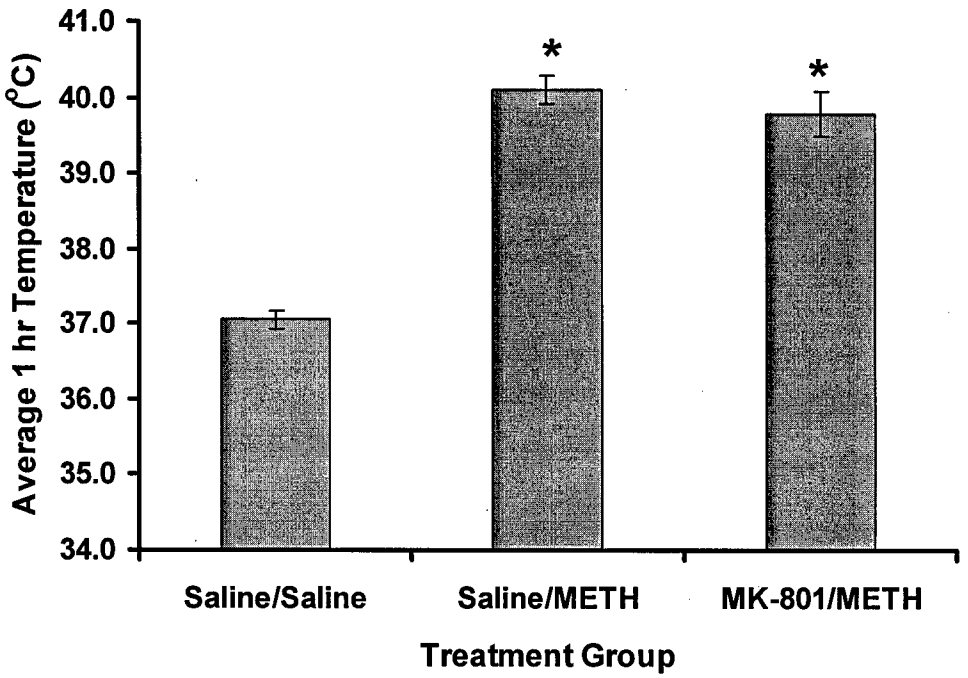

DAT Density (% of Saline Control)
120
100
80
60
40
20
0
*
*
Saline/Saline
Saline/METH
MK-801/METH
Treatment Group
78 kDa

Fig. 7


41.0
40.0
39.0
38.0
37.0
36.0
35.0
34.0
Average 1 hr Temperature (°C)
*
*
Saline/Saline
Saline/METH
MK-801/METH
Treatment Group

Psychopharmacology (●) : 1–10
DOI 10.1007/s00213-005-0007-6

APPENDIX 7

ORIGINAL INVESTIGATION

Susan L. Broom · Bryan K. Yamamoto

Effects of subchronic methamphetamine exposure on basal dopamine and stress-induced dopamine release in the nucleus accumbens shell of rats

Received: 24 August 2004 / Accepted: 15 March 2005

Abstract *Rationale:* Subchronic administration of stimulants reduces basal dopamine (DA) concentrations and blocks stress-induced DA release in the nucleus accumbens (NA) of rats during withdrawal. However, no studies have attempted to relate early withdrawal from chronic drug exposure to stress reactivity and changes in DA transmission. *Objectives:* The effects of subchronic low-dose methamphetamine (METH) administration on regional changes in dopamine transporter (DAT) and norepinephrine transporter (NET) immunoreactivity and function during early withdrawal were examined. The effects of subchronic METH on stress responsivity measured by DA release in the nucleus accumbens shell (NA SHELL) and core (NA CORE) during acute restraint stress were also examined. *Methods:* Male rats received single injections of METH (2.0 mg/kg i.p.) or saline (SAL) for 10 days and then were killed 24 h after the last injection. DAT and NET protein in NA, striatum (STR), medial prefrontal cortex (mPFC), and hippocampus were assayed by Western blot analysis. Experiment 2 measured basal extracellular DA concentrations and restraint-stress-induced DA release in vivo in the NA SHELL and CORE of SAL- and METH-pretreated rats after 24-h withdrawal. Experiment 3 examined the in vivo regulation of extracellular DA in the NA SHELL and/or CORE after local administration of GBR12909 (50 μM) or nisoxetine (100 μM; NA SHELL). *Results:* Subchronic METH increased DAT but not NET immunoreactivity in the NA compared to the STR and mPFC. METH reduced basal extracellular DA and blocked restraint-stress-induced DA release in the NA SHELL. DA uptake blockade increased extracellular DA more in the NA SHELL of METH rats, whereas NE uptake blockade increased basal DA concentrations to a similar extent in METH and SAL rats. *Conclusions:* These results suggest that subchronic METH exposure selectively increases NA DAT and consequently reduces basal and stress-induced DA release in the NA SHELL during early withdrawal.

Keywords Psychostimulants · Nucleus accumbens · Dopamine transporter · GBR12909 · Norepinephrine transporter · Nisoxetine · Stress · In vivo microdialysis · Western blot

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Introduction

Methamphetamine (METH) is a psychostimulant that activates mesolimbic regions associated with drug reward. METH exerts its dopamine (DA)-, norepinephrine (NE)-, and serotonin (5-HT)-releasing effects primarily through reversal of their respective plasmalemmal transporters. The activation of the dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NA) is the major feature of abused drugs associated with euphoria (Robinson and Berridge 1993; Leshner and Koob 1999; Koob and Le Moal 2001) and high abuse potential. Clinical research also suggests that long-term METH exposure affects mesolimbic DA function, as measured through increased glucose metabolism in the ventral striatum of abstinent METH abusers (London et al. 2004).

Acute exposure to psychostimulants (Pontieri et al. 1995) and stress (Kalivas and Duffy 1995) selectively increase DA release in the nucleus accumbens shell (NA SHELL), suggesting that DA release after stimulant and stress exposure differs among NA subregions. In fact, increased DA release in the NA SHELL, as opposed to the nucleus accumbens core (NA CORE), mediates the euphoric properties of abused psychostimulants (Pontieri et al. 1995; Sellings and Clarke 2003) and may serve as a coping strategy in response to stress challenges (Kalivas and Duffy 1995). In addition, acute stress exposure to humans can affect the subjective effects of METH, enhance drug craving (Soderpalm

et al. 2003), and possibly contribute to METH abuse and relapse.

Whereas previous studies show that chronic administration of psychostimulants enhance DA responsiveness to subsequent drug challenge (i.e., sensitization), others have shown that basal extracellular DA concentrations in NA SHELL were significantly *reduced* after chronic administration of opiates, psychostimulants, or ethanol self-administration in rats (Rossetti et al. 1992; Gerrits et al. 2002). Moreover, recent evidence indicates that exposure to a chronic low-dose *d*-amphetamine regimen reduces basal and restraint-stress-induced DA concentrations in the NA of rats during a 12-h and 7-day withdrawal period (Weiss et al. 1997). These data suggest that chronic low-dose drug exposure may dysregulate the mesolimbic DA system, producing negative affect and/or a compromised responsivity to a stress challenge. However, no previous studies have attempted to understand the underlying mechanisms that relate chronic drug exposure to stress reactivity and changes in DA transmission.

We hypothesized that chronic low-dose administration of METH would decrease basal extracellular concentrations of DA and attenuate the stress-induced increases in DA in the NA SHELL but not the NA CORE. Because both DAT (Pierce and Kalivas 1997) and NET (Yamamoto and Novotney 1998) regulate extracellular DA in the NA SHELL, it is hypothesized that the reduced basal DA in the NA SHELL during early withdrawal from chronic METH results from increased DAT and/or NET protein expression and increased transporter-mediated regulation of extracellular DA concentrations in vivo.

Materials and methods

Male Sprague–Dawley rats (175–200 g upon arrival) were habituated to the colony room for 2–3 days followed by 2–3 days of handling in the colony room before experimental manipulations. Rats were housed on a 12-h light/dark cycle (lights on at 0700 hours), with all experiments and procedures conducted during the light cycle. On each day of METH exposure (10 consecutive days), the researcher transported the rats from the colony to the test room. Rats received an intraperitoneal (i.p.) injection in the home cage of either *d*-METH (2 mg/kg) or saline (SAL; 1 ml/kg). Dose was determined based on weight of the salt. This procedure allowed for preexposure to the test room and reduced environmental and experimenter-related novelty stress. All procedures were conducted in accordance to the *Guide for the Care and Use of Laboratory Animals*, and all procedures were approved by the Boston University School of Medicine Institutional Animal Care and Use Committee.

DAT and NET Western blot analyses

Separate groups of rats received subchronic METH regimens and then killed at the 24-h withdrawal period for DAT and NET protein content in the striatum (STR), medial prefrontal cortex (mPFC), NA, and hippocampus. Nucleus accumbens tissues were pooled from two animals. Tissues were homogenized and synaptosomes prepared as described previously (Fleckenstein et al. 1997). Protein concentrations were determined via the method of Bradford. Western blot analysis was performed using 9–10 μg of protein loaded onto a 10% Tris–glycine gel. Electroblotting of proteins to PVDF membranes were carried out in 1× Tris–glycine transfer buffer containing 20% methanol and 0.02% SDS. Membranes were blocked with a 5% milk solution and incubated overnight at 4°C with primary antibody specific for DAT (1:1000 STR; 1:500 NA, mPFC; Santa Cruz) or NET (1:500 Chemicon). The DAT blots were washed four times in 1× PBS and incubated with alkaline phosphatase conjugated rabbit anti-goat secondary antibody (Santa Cruz) for 1 h. The NET blots were washed four times in 1× PBS and incubated with alkaline phosphatase-conjugated goat anti-rabbit (Chemicon) secondary antibody for 1 h. All blots were normalized to actin (Chemicon). Alkaline phosphate was visualized using a chromagenic solution derived from BCIP and NBT (Promega) and net band optical densities were quantified by a Kodak Gel Logic 100 imaging system. Data are graphically presented as percent saline optical densities.

Surgical procedure

Surgical procedures occurred on the 7th day of METH treatment. This time frame was selected to allow for a 3-day recovery period between surgery and dialysis. Rats were anesthetized with an i.p. injection of ketamine (10 mg/kg) and xylazine (6 mg/kg). Stainless steel guide cannulae (21 gauge) were implanted and positioned at 1.7 mm anterior to bregma and 1.1 mm lateral to bregma at a 15° angle for the NA SHELL. For the NA CORE, guide cannulae were implanted at 1.7 mm anterior to bregma and 2.0 mm lateral to bregma (Paxinos and Watson 1986). The guide cannulae were secured to the skull with three stainless steel screws and cranioplast cement. Rats were returned to the colony room after the surgery.

In vivo microdialysis

Microdialysis experiments were conducted in separate groups of rats at 24 h after the last METH injection. On the day of dialysis, rats were transported to the test room and microdialysis probes were lowered through the guide cannula exactly 8 mm below the skull surface for both NA SHELL and NA CORE groups. The active membrane extended 2.0 mm beyond the stainless steel barrel. This distance was predetermined by a 5-mm PE50 sleeve covering a 1-mm PE20 section glued on the stainless steel barrel at a distance of 17 mm from the barrel tip to act as a "stop." The dialysis line was perfused with modified Dulbecco's phosphate-buffered saline (138 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 1.2 mM $CaCl_2$, 5.0 mM glucose, pH 7.4). The flow rate was set at

2 μl/min and baseline samples were collected after a 1.5-h equilibration period. All samples were collected at 30-min intervals. For the first experiment, three samples each were collected for baseline (1.5 h), during restraint stress (1.5 h), and after release from restraint (1.5 h). During restraint stress, rats were removed from the dialysis chamber and placed with their ventral surface on a Plexiglas board. The rats were secured to the board with two 2-in. Velcro straps, one located across the lower midregion and the other just behind the head (Matuszewich et al. 2002). The paws were secured with tape. Rats were released after the stress collection period and returned to the dialysis chambers for the remainder of the sampling period (1.5 h). For the second and third experiments, four samples each were collected for baseline (2 h), during drug perfusion (2 h), and post drug perfusion (2 h). For Experiment 2, GBR12909 (50 μM; Sigma Chemical Co.) was reverse dialyzed during the 2-h drug perfusion period. This concentration was selected based on previous work in this laboratory (B.K. Yamamoto, unpublished data) and others demonstrating that it is within the dose range known to increase extracellular DA concentrations in the NA SHELL (Rahman and McBride 2000; Engleman et al. 2000; Pierce and Kalivas 1997). For Experiment 3, nisoxetine (Sigma Chemical Co.; 100 μM) (Li et al. 1996) was reverse dialyzed during the drug perfusion period. A 20-μl aliquot was analyzed for DA concentrations by high-performance liquid chromatography coupled with electrochemical detection (HPLC-EC) as previously described (Nash and Yamamoto 1992). Peak separation was determined using a 3 μm C-18 column (100×2 mm) and a mobile phase containing 32 mM citric acid, 54.3 mM sodium acetate, 0.074 mM Na_2 EDTA, 0.22 mM octyl sodium sulfate, and 5% methanol (pH 4.2). The oxidation potential was set to 0.65 mV, with a sensitivity of 0.5–1.0 nA and a flow rate of 0.200 ml/min. Data are reported as pg/20 μl dialysate. In a separate experiment, tetrodotoxin (TTX; 1.0 μM) was reversed dialyzed into the NA CORE (n=6). Extracellular DA concentrations were measured every 30 min for 1.5 h before TTX administration and continued for 3.5 h throughout TTX administration.

Histological analyses

In all experiments, rats were killed by rapid decapitation 24 h after the dialysis experiment. Brains were removed and rapidly frozen on dry ice. Brains were sectioned on a cryostat (−20°C) and probe placements recorded. Only placements within NA CORE or NA SHELL were used for subsequent data analyses (see Fig. 1).

Statistical analyses

Two-way, repeated measures analyses of variance (ANOVAs) were used to compare rats pretreated with METH to rats pretreated with SAL across all samples, including experiments that involve the local perfusion of drug and restraint-stress procedures. Tukey's HSD post hoc tests were used to determine significant interactions. For the TTX experiment, a repeated measures ANOVA was used to assess changes in extracellular DA concentrations over time during the TTX perfusion. Comparisons between individual time points were made using Tukey's HSD post hoc tests. The Western blot data were analyzed using t tests. Statistical significance was set at $p<0.05$ for all conditions. Data are reported as mean±SEM.

Results

Effects of subchronic METH administration on DAT protein immunoreactivity

Experiment 1 examined the effects of subchronic METH exposure (2.0 mg/kg for 10 days) on dopamine transporter (DAT) protein immunoreactivity in the STR, mPFC, and NA of rats. DAT immunoreactivity appeared in the 64- to 98-kDa range. Dopamine transporter immunoreactivity (percent SAL optical density) as a function of brain region is presented in Fig. 2. On average, METH-pretreated groups showed DAT immunoreactivity values at 99±6.45% of SAL rats (100±6.16%) for the STR (n=13–14 per group); 96.95±10.19% of SAL rats (100±5.29%) for the mPFC (n=8 per group); and 150±20.48% of SAL rats (100±11.28%) for the NA (n=7 per group). METH pretreatment produced a significant increase in NA DAT [$t(1,12)=2.365$, $p<0.05$], with no significant differences in STR and mPFC noted between SAL- and METH-pretreated groups.

Effects of subchronic METH administration on NET protein immunoreactivity

Experiment 1 also measured the effects of subchronic METH exposure (2.0 mg/kg for 10 days) on NET immunoreactivity in the mPFC, hippocampus (HIPP), and NA of rats. NET immunoreactivity appeared between 50 and 64 kDa. On average, METH-pretreated rats showed NET immunoreactivity values at 104±7.62% compared to SAL rats (100±8.68%) for the mPFC (n=8 per group); 85±11.91% of SAL rats (100±12.73%) for the HIPP (n=8–9 per group); and 85.68±9.21% of SAL rats (100±8.27) for the NA (n=7–8 per group). Despite lower NET immunoreactivity values observed in METH-pretreated groups in the HIPP and NA, t tests on each region revealed no significant changes in NET immunoreactivity.

Effects of subchronic METH administration on basal extracellular concentrations of DA and restraint-stress-induced DA release in the NA SHELL and NA CORE

Extracellular concentrations of DA in the NA SHELL (pg/20 μL) as a function of time (minutes) are presented in Fig. 3. Basal extracellular concentrations of DA across three baseline samples in the NA SHELL were higher (2.61±0.18 pg/20 μL) for SAL-pretreated (n=5) than METH-

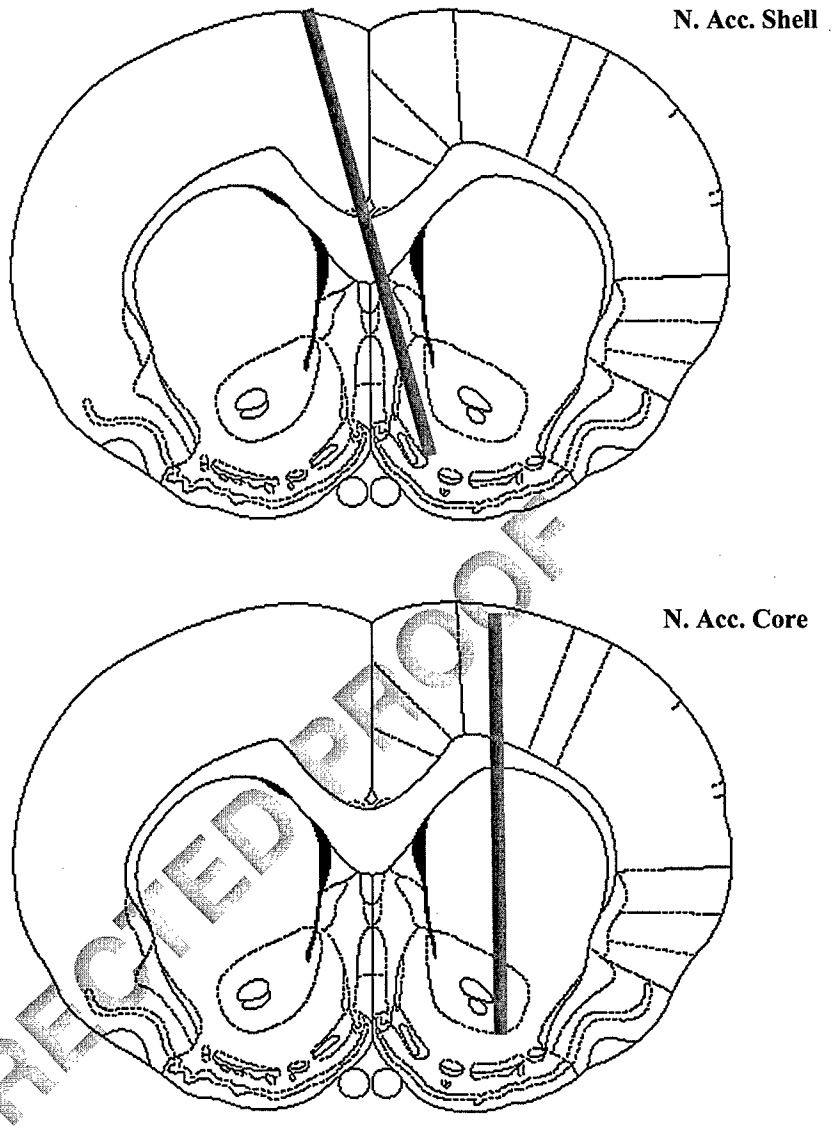

Fig. 1 Representative template of probe placements used for nucleus accumbens shell (*N. Acc. Shell*) and nucleus accumbens core (*N. Acc. Core*) in vivo microdialysis experiments

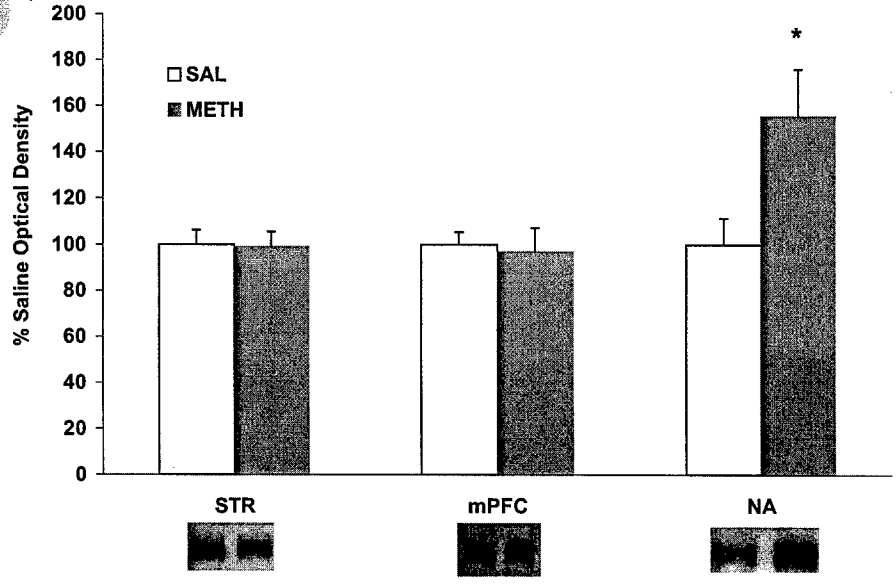

Fig. 2 DAT immunoreactivity in the striatum (*STR*), medial prefrontal cortex (*mPFC*) and nucleus accumbens (*NA*) of SAL (*open bars*) and METH (*filled bars*) pretreated rats. DAT immunoreactivity appeared in the 64- to 98-kDa range. Data are presented as percent of saline optical densities. n=7–14. METH-pretreated rats showed significantly higher DAT (*p<0.05) in the NA as compared to SAL-pretreated rats

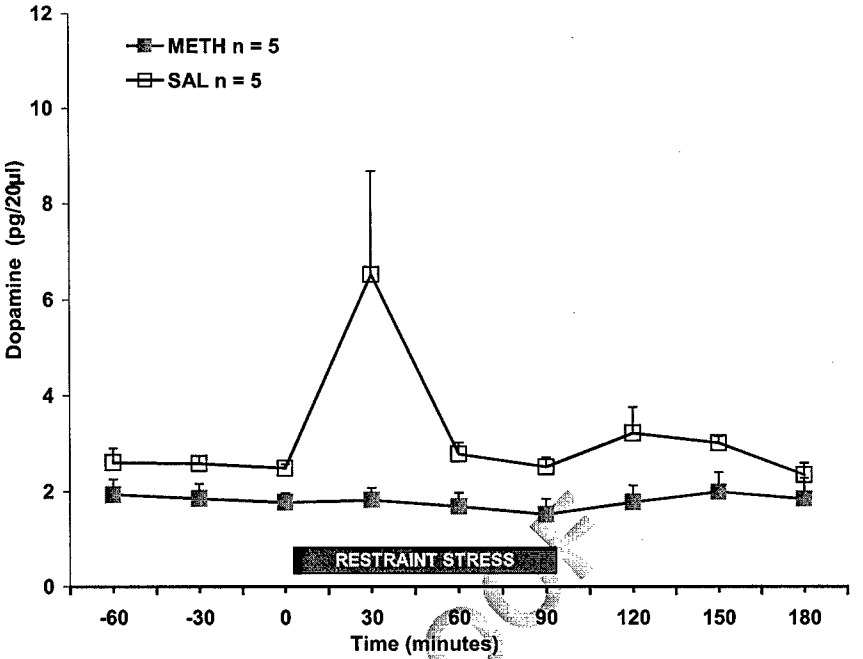

Fig. 3 Basal and restraint-stress-induced DA concentrations (pg/20 µl) in the NA SHELL for SAL- (*open squares*) and METH- (*filled squares*) pretreated rats. The *solid bar* indicates the time of restraint stress. METH pretreatment significantly reduced extracellular DA concentrations compared to SAL-pretreated rats ($p<0.05$). Extracellular DA concentrations over time were significantly increased during exposure to restraint stress ($p<0.01$); however, this increase was blocked in METH-pretreated rats (Pretreatment×Time interaction; $p<0.01$). $n=5$

pretreated rats (1.86±0.38 pg; $n=5$; Fig. 3). Restraint stress produced an approximate 2.5-fold increase in NA SHELL DA of SAL-pretreated rats (6.52±2.15 pg/20 µL) during the initial sampling period after restraint stress, whereas no increase was observed in METH-pretreated rats during restraint stress (1.82±0.34 pg/20 µL). SAL-pretreated rats showed higher extracellular DA concentrations in the NA SHELL for all remaining stress samples and samples collected after the restraint stress. METH pretreatment significantly reduced extracellular DA concentrations compared to SAL-pretreated rats [main effect of pretreatment: $F(1,8)=6.78$, $p<0.05$]. Extracellular DA concentrations over time were significantly increased during exposure to restraint stress [main effect over time; $F(8,8)=3.21$, $p<0.01$]; however, this increase was blocked in METH-pretreated rats [Pretreatment×Time interaction; $F(8,64)=3.09$, $p<0.01$].

Basal extracellular concentrations of DA across the three baseline samples in the NA CORE averaged 1.50±0.25 pg/20 µL for SAL-pretreated rats ($n=6$) and 1.80± 0.34 pg/20 µL for METH-pretreated rats ($n=5$). METH pretreatment did not significantly change extracellular DA in the NA CORE compared to SAL-pretreated rats. Extracellular DA concentrations for both groups differed statistically over time [$F(8,9)=2.59$, $p<0.02$], with a significant Pretreatment×Time interaction [$F(8,72)=2.48$, $p<0.02$]. Despite the statistical differences noted, post hoc tests conducted on samples combined into baseline, stress, and poststress sampling blocks revealed no relevant differences between SAL- and METH-pretreated rats over the observation period. For the TTX experiment ($n=6$), DA concentrations in the NA CORE were significantly decreased over time during the TTX perfusion ($F(9,45)=114.61$, $p<0.001$). Basal concentrations before the TTX perfusion averaged 3.82 + 0.14 pg/20 µl. Local administration of TTX (1 µM) into the NA CORE produced significant decreases in extracellular DA concentrations during the first 30 min (2.62±0.23 pg/20 µl; $q=7.74$, $p<0.001$). DA concentrations continued to decrease over a 2.5-h collection period to 1.88±0.16, 1.18±0.10, 0.77±0.08, 0.43±0.05, and 0.22±0.03 pg/20 µl at the 1-, 1.5-, 2-, 2.5-, and 3-h time points, respectively. No further detectable decrease occurred at the 3.5-h time point during the TTX perfusion compared to the 3-h time point (0.22± 0.03 pg/20 µl vs 0.15±0.04 pg/20 µl). These results demonstrate that impulse-dependent changes in extracellular DA were being examined.

Effects of local administration of GBR12909 on basal extracellular concentrations of DA in the NA SHELL and NA CORE in SAL and METH-pretreated rats

Experiment 2 measured the effects of subchronic administration of METH (2.0 mg/kg i.p. for 10 days) on basal extracellular DA concentrations after local administration of the DAT inhibitor GBR12909 (50 µM). Extracellular concentrations of DA in the NA SHELL (pg/20 µL) as a function of time (minutes) before and after GBR12909 for METH ($n=5$) and SAL ($n=6$) pretreated rats are presented in Fig. 4. Basal extracellular concentrations of DA in the NA SHELL averaged 1.25±0.27 pg/20 µL for SAL-pretreated rats and 1.00±0.13 pg/20 µL for METH-pretreated rats across the first three baseline samples. The fourth baseline sample was not included because the sample also contained a small amount of dialysate treated with GBR12909. GBR12909 administration increased basal extracellular DA concentrations in both groups. SAL rats showed a sustained two- to threefold elevation in extracellular DA throughout the perfusion period and METH-pretreated rats showed an approximate tenfold increase in the initial sampling period that returned toward basal concentrations during the two

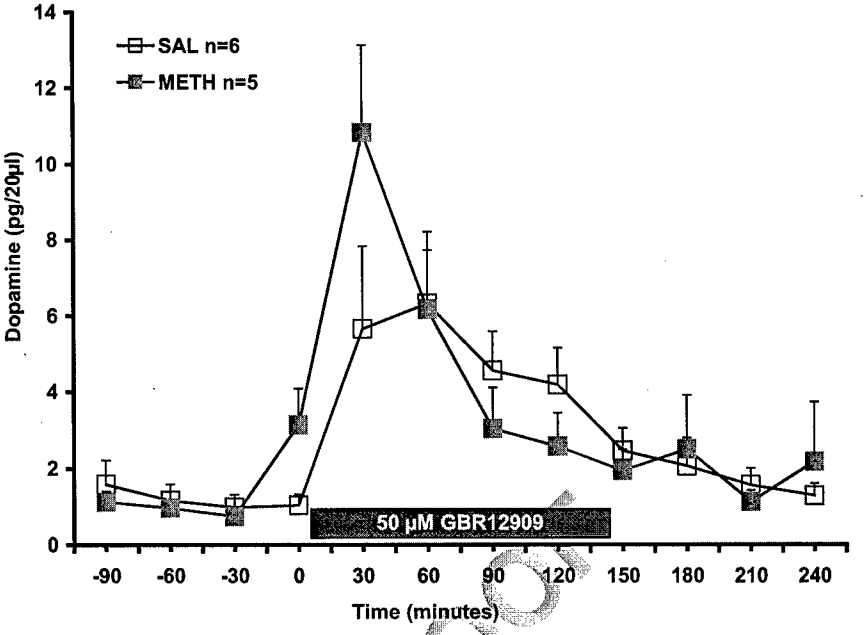

Fig. 4 Basal extracellular concentrations of DA in the NA SHELL for SAL- (*open squares*) and METH- (*closed squares*) pretreated rats after local perfusion of GBR12909 (50 µM). *Solid line* indicates samples collected during GBR12909. Basal extracellular DA concentrations were the same across the first three baseline samples for SAL- and METH-pretreated rats; however, significantly higher basal extracellular DA concentrations after GBR12909 administration ($p<0.01$) were observed in both groups, and the time course between METH- and SAL-pretreated rats with regard to extracellular DA concentrations during GBR12909 perfusion was significantly different (Pretreatment×Time interaction; $p<0.05$). n=5–6

remaining samples collected in the presence of GBR12909 perfusion. METH pretreatment alone did not significantly affect basal extracellular DA concentrations across the observation period. Rather, significantly higher basal extracellular DA concentrations after GBR12909 administration [main effect of time; $F(1,3)=6.124$, $p<0.01$] were observed in both groups, and the time course between METH- and SAL-pretreated rats with regard to extracellular DA concentrations during GBR12909 perfusion was different [Pretreatment×Time interaction; $F(9,27)=6.124$, $p<0.05$].

Basal extracellular concentrations of DA in the NA CORE were similar across the four baseline samples for SAL-pretreated rats (2.82±0.54 pg/20 µL; n=5) and METH-pretreated rats (3.10±0.48 pg/20 µL; n=6). METH pretreatment had no effect on basal extracellular DA concentrations in the NA CORE. Baseline samples for both groups decreased over time [$F(3,9)=3.26$, $p<0.01$]; however, no significant Pretreatment×Time interaction was noted.

Local GBR12909 administration resulted in an approximate 1.5-fold increase in basal extracellular DA concentrations METH-pretreated rats and an approximate 2.5-fold increase in SAL-pretreated rats in the first GBR12909 sample after baseline four. The pattern of response appeared to differ among pretreated groups over the drug perfusion period. SAL rats tended to show more sustained elevations in extracellular DA concentrations, whereas METH rats tended to decrease in extracellular DA concentrations during the remaining samples collected during GBR12909 perfusion. METH pretreatment had no effect on extracellular DA concentrations in response to GBR12909 perfusion. However, both pretreatment groups showed increases in extracellular DA concentrations from the fourth baseline sample [$F(4,9)=3.75$, $p<0.02$]; no Pretreatment×Time interaction was noted.

Effects of local administration of nisoxetine on basal extracellular concentrations of DA in the NA SHELL in SAL- and METH-pretreated rats

Experiment 3 evaluated the effects of subchronic administration of METH (2.0 mg/kg i.p. for 10 days) on basal extracellular DA concentrations in the NA SHELL after local administration of the NET inhibitor nisoxetine (NIS; 100 µM). Extracellular concentrations of DA in the NA SHELL (pg/20 µL) as a function of time (minutes) for SAL- (n=7) and METH- (n=5) pretreated rats are presented in Fig. 5. Basal DA values across four baseline samples were higher in SAL-pretreated rats (2.53±0.45 pg/20 µL) than METH-pretreated rats (1.38±0.18 pg/20 µL). The baseline samples were more variable in SAL rats than METH-pretreated rats. Therefore, to reduce variability and increase power, three rats (two SAL and one METH) that contributed baseline data (but were not treated subsequently with NIS due to perfusion failures in the later part of the experiment) were added to the treatment groups. METH-pretreated rats (1.28±0.16 pg/20 µL) showed significantly reduced basal DA concentrations [$t(1,58)=2.43$; $p<0.03$] in the NA SHELL compared to SAL-pretreated rats (2.35± 0.34 pg/20 µL). Both groups showed an approximate two- to threefold increase in basal extracellular DA concentrations during the local perfusion of NIS into the NA SHELL. METH pretreatment alone had no effect on extracellular DA concentrations during NIS perfusion. Both METH- and SAL-pretreated rats showed a significant increase in extracellular DA concentrations from the fourth baseline sample during the NIS perfusion period [main effect of time $F(4,10)=7.10$, $p<0.01$]; no significant Pretreatment×Time interaction was noted.

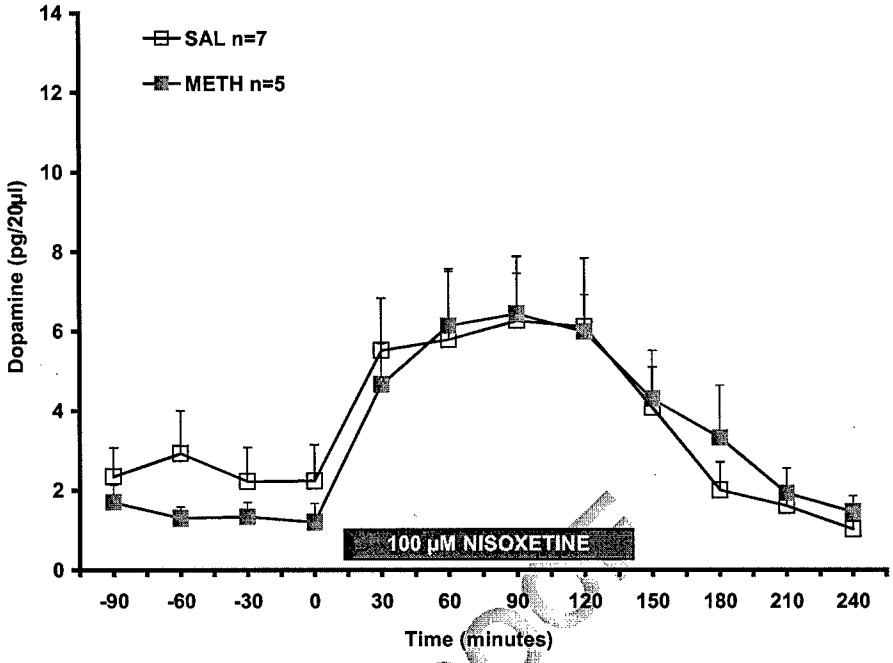

Fig. 5 Basal extracellular concentrations of DA in the NA SHELL in SAL- (*open squares*) and METH- (*closed squares*) pretreated rats after local administration of nisoxetine (100 µM). *Solid line* indicates samples collected during nisoxetine. Basal extracellular DA concentrations across the four combined baseline samples were significantly lower in METH-pretreated rats compared to SAL-pretreated rats ($p<0.03$; see Results). NE uptake blockade significantly increased basal DA concentrations ($p<0.01$) compared to the fourth baseline sample for both METH- and SAL-pretreated groups. n=5–7

Discussion

This study examined the effects of subchronic administration of low doses of METH on selective regional changes in DAT and NET immunoreactivity and function during early withdrawal. In addition, the effects of subchronic METH on stress responsivity with respect to DA release in the NA SHELL and CORE during an acute restraint stress episode were examined. Subchronic METH exposure selectively increased DAT but not NET immunoreactivity in the NA compared to the STR and mPFC. Basal extracellular DA concentrations were reduced and restraint-stress-induced DA release in the NA SHELL (but not NA CORE) was blocked in METH-pretreated rats. Furthermore, DA uptake blockade increased the extracellular concentrations of DA more in the NA SHELL of METH-pretreated rats, whereas NE uptake blockade increased basal extracellular DA concentrations to a similar extent in METH- and SAL-pretreated rats.

The finding that subchronic administration of METH produced a significant increase in NA DAT immunoreactivity (Fig. 2) when measured 1 day after the drug administration regimen is consistent with the hypothesis that chronic administration of METH reduces basal extracellular DA concentrations in the NA during early withdrawal. Subchronic self-administration of cocaine (Arroyo et al. 2000) or subchronic systemic administration of *d*-amphetamine (2.5 mg/kg i.p. for 5 days; Shilling et al. 1997) increased DAT mRNA in the VTA during 7- to 10-day withdrawal. However, others have reported that DAT densities (as measured through quantitative autoradiography) in NA SHELL and NA CORE were unchanged after 7- to 30-day withdrawal from METH administration in rats (Stefanski et al. 2002), or decreased in the NA after a 10-day withdrawal period from cocaine (Sharpe et al. 1991; Cerruti et al. 1994; Pilotte et al. 1996). Recent studies demonstrate that *acute* application of amphetamine to human dopamine transporter (hDAT) in EM4 cells produced hDAT internalization (Saunders et al. 2000). It is likely that these discrepancies in the changes in DAT can be explained by differences in the type of psychostimulant studied, the type of administration paradigm (self vs passive), and the durations of exposure and withdrawal. Regardless, no previous studies have attempted to relate changes in DAT density, protein, or mRNA produced by subchronic stimulant exposure with transporter function and dynamic changes in extracellular dopamine. Along these lines, the present study showed that the subchronic METH exposure increases DAT immunoreactivity in the NA and that these increases are associated marked changes in extracellular concentrations of DA during basal conditions and in response to an acute stressor or dopamine uptake blockade.

DAT immunoreactivity after an early withdrawal period is significantly increased in the NA, whereas basal DA concentrations in the NA SHELL (Figs. 3 and 5) are reduced. Thus, it is possible that the increases in DAT protein may account for the enhanced uptake and lower basal concentrations of extracellular DA. The enhanced uptake of DA by NET in the NA SHELL may also contribute to the observed decreases in extracellular DA in METH-pretreated rats due to the heterotransport of DA by NET in this subregion (Yamamoto and Novotney 1998). However, this possibility is unlikely due to the finding that subchronic METH exposure did not increase NET immunoreactivity in the NA (see Results). It should be noted that we did not distinguish DAT and NET immunoreactivities in the NA SHELL and NA CORE subregions of the NA. Moreover, since NET is localized almost exclusively to the NA SHELL (Berridge et al. 1997; Delfs et al. 1998) and the

changes in basal extracellular DA were restricted to this subregion and not the NA CORE, the decreases in basal extracellular DA after subchronic METH are likely the result of increases in DAT and not NET protein in the NA SHELL.

These results also illustrate that not all dopaminergic terminal brain regions are affected equally by subchronic METH exposure. METH exposure selectively increased DAT in the NA compared to the STR and mPFC (Fig. 2) and did not significantly change NET immunoreactivity in the mPFC, hippocampus, or NA of rats during early withdrawal. These studies further illustrate that the mesolimbic DA system, and in particular the mesoaccumbens DA system, is selectively affected by chronic METH (London et al. 2004).

The brain-region-selective effect of subchronic METH administration is further delineated within the mesoaccumbens DA system. Although basal extracellular DA was decreased by subchronic METH in the NA SHELL (Figs. 3 and 5), no effects were observed in the NA CORE (see Results). Moreover, these data are consistent with others showing that acute stress exposure selectively increases DA in the NA SHELL but not NA CORE (Kalivas and Duffy 1995) and extend these findings by showing that subchronic METH exposure does not change basal DA or stress-induced DA release in the NA CORE during early withdrawal. This is consistent with previous research indicating that subchronic exposure to amphetamine or cocaine reduces basal concentrations of DA in the NA (Rossetti et al. 1992; Weiss et al. 1992, 1997; Gerrits et al. 2002), in addition to blocking stress-induced DA release in the NA of rats (Weiss et al. 1997). Although some suggest that basal DA does not change in the NA during later withdrawal (20–22 days) from chronic cocaine exposure (Pierce and Kalivas 1995; Hooks et al. 1994), we did not measure DAT immunoreactivity in the NA during longer withdrawal periods. Therefore, we cannot rule out the possibility that DAT protein returns to control levels during longer withdrawal periods in METH-exposed rats to compensate for the reduction in extracellular DA in the NA SHELL that occurs during early withdrawal. Regardless, our findings suggest that subchronic stimulant exposure increases NA DAT immunoreactivity to differentially reduce basal and stress-induced release of DA in the NA SHELL (as opposed to NA CORE) during early withdrawal.

The effects of local GBR12909 perfusion in the NA CORE and NA SHELL were also different and suggest that not only is there a preexistent differential effect on extracellular DA in response to DAT blockade in NA SHELL compared to NA CORE, but that subchronic METH exposure selectively altered the DAT-dependent regulation of extracellular DA in the NA SHELL (Fig. 4). Local perfusion of GBR12909 produced an approximately tenfold increase in basal DA concentrations in the NA SHELL of METH-pretreated rats, whereas a more sustained two- to threefold extracellular increase in NA SHELL DA concentrations was observed in SAL-pretreated rats during DAT blockade (Fig. 4). The results from SAL-pretreated rats are consistent with others showing increased extracellular DA concentrations in the NA after DAT blockade (Pierce and Kalivas 1997; Engleman et al. 2000; Rahman and McBride 2000) and STR (Nakachi et al. 1995; Nomikos et al. 1990). In contrast, DAT blockade in the NA CORE resulted in less substantial increases in extracellular DA concentrations in both METH- and SAL-pretreated groups. These data are consistent with previous research indicating more robust effects of cocaine sensitization on extracellular DA concentrations in the NA SHELL vs the NA CORE during early withdrawal (Pierce and Kalivas 1995). Our results on early withdrawal from chronic METH exposure also extend previous findings on cocaine-induced DA release showing that DAT in the NA SHELL compared to the NA CORE is more efficient at clearing extracellular DA (David et al. 1998).

Results from Experiment 2 (Fig. 4) suggest that there is differential regulation of basal extracellular DA concentrations in the NA SHELL after a 24-h withdrawal from subchronic METH exposure. The finding that blockade of DAT after subchronic low-dose METH exposure results in *enhanced* increases in extracellular DA concentrations in the NA SHELL is consistent with the augmented increases in basal extracellular DA concentrations observed during local administration of GBR12909 in chronic cocaine-exposed rats (Pierce and Kalivas 1997). It is important to note that although we have repeatedly observed decreases in basal extracellular DA after chronic METH (Figs. 3 and 5), reduced basal DA concentrations in the NA SHELL were not observed when ethanol was used in the perfusion medium as the vehicle control during baseline before the perfusion of GBR12909 (Fig. 4). It is possible that the low concentration of ethanol increased basal DA concentrations of METH-pretreated rats to normal control values (Weiss et al. 1996) but did not affect the saline-pretreated groups (Yim et al. 1998) and consequently obscured the decreases in basal extracellular DA produced by subchronic METH.

The more rapid return to basal concentrations of DA in the NA SHELL of METH-exposed rats after local administration of the DAT inhibitor GBR12909 (Fig. 4) suggests that other mechanisms are involved in regulating extracellular DA concentrations in the absence of DAT. Two potential explanations for the differential pattern of extracellular DA concentrations after DAT inhibition involve D2 dopamine autoreceptor sensitivity and heterotransporter regulation of extracellular DA. The rapid return to basal DA concentrations in the NA SHELL of METH-exposed rats could result from hypersensitive D2 autoreceptors in the VTA, which on activation would rapidly decrease impulse-mediated DA release (Amano et al. 2003). However, other evidence shows that decreases in D2 autoreceptors in the VTA and D1 receptors in the NACC are only observed in rats after voluntary chronic self-administration of METH and not after chronic yoked administrations of METH (Stefanski et al. 1999). We did not assess whether subchronic METH exposure increases D2 autoreceptor sensitivity in the VTA; however, our data do support the hypothesis that subchronic METH exposure significantly changes the dynamic regulation of extracellular DA after DA uptake blockade in vivo in the NA. It is possible that the observed

rapid decline in extracellular DA, despite the continued presence of uptake blockade, is due to an abrupt decrease in impulse-mediated DA release resulting from increased D2 autoreceptor sensitivity produced by chronic METH. The possibility of reduced tyrosine hydroxylase after chronic METH exposure is unlikely, given that the significant elevation in extracellular DA during DA uptake blockade are observed in METH-pretreated rats compared to SAL-pretreated rats. In addition, recent research indicates that tyrosine hydroxylase activity in the NA SHELL or NA CORE does not change after early (24 h) or late (14 day) withdrawal from subchronic cocaine (Licata and Pierce 2004).

The finding that the local administration of the NET inhibitor nisoxetine increased basal extracellular concentrations of DA in the NA SHELL (Fig. 5) is consistent with previous work showing that local administration of the NET inhibitor, desipramine, increased extracellular DA concentrations in the NA SHELL of rats in vivo (Yamamoto and Novotney 1998). Although some studies have shown that NET binding increased in the bed nucleus stria terminalis of chronic cocaine-exposed nonhuman primates (Macey et al. 2003) and that norepinephrine is involved in stress-induced drug relapse (Shaham et al. 2000; Erb et al. 2000; Stewart 2000; Leri et al. 2002), no studies have examined the effects of subchronic METH exposure on NET protein immunoreactivity in the NA. The present study shows that the decreases in basal extracellular DA in the NA SHELL produced by subchronic METH are not due to the enhanced activity of NET (Fig. 5) or an increase in NET protein.

In summary, these data illustrate that subchronic METH exposure selectively increases DAT but not NET immunoreactivity in the NA of rats. Moreover, subchronic METH exposure selectively reduces DA function in the NA SHELL compared to the NA CORE as evidenced by reduced basal DA concentrations, a blockade of stress-induced DA release, and an augmented but short-lived responsiveness to DAT uptake blockade in the NA SHELL. These findings have significance for understanding how repeated METH administrations selectively change mesoaccumbens DA transmission during early withdrawal and the ability to cope with stress. Given that reduced mesolimbic DA function is associated with negative affective disorders such as drug craving (Koob and Le Moal 1997), these data may also explain why a considerable population of METH abusers relapse during early withdrawal (Brecht et al. 2000, 2004).

Acknowledgements This project was supported by the National Institute on Drug Abuse grants DA07606 and Department of Defense DAMD17-99-1-9479.

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